Stachydrine and Synephrine, a Pair of Bioactive Equilibrists in Four Chinese Herbs from *Citrus* Genus

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Article

Stachydrine and Synephrine, a Pair of Bioactive Equilibrists in Four Chinese Herbs from *Citrus* Genus

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Abstract: Four Chinese herbs *Zhishi*, *Zhiqiao*, *Qingpi* and *Chenpi* from *Citrus* genus were widely used for treating various cardiovascular and gastrointestinal diseases. Although many pharmacological functions of these herb decoctions can be clarified from the bioactivities of identified ingredients, some of them remain confusing. To clarify the reasons of those confusing functions, depending on the extended structure-activity relationships of cholinergic and anti-cholinergic agents, here a simple method for quickly discovering possible choline analogs was established using a specific TLC method, and then stachydrine and choline were first identified from these *Citrus* herb decoctions based on their NMR and HRMS data. After this, two TLCS methods were first established respectively for the quantitatively analyses of stachydrine and choline, and the contents of both two ingredients and synephrine in 39 samples were determined using the valid TLCS and HPLC methods, respectively. The results showed the contents of stachydrine were 2.4 times to those of synephrine in *Zhiqiao* while about 1/3 to 2/3 in *Zhishi*, *Qingpi* and *Chenpi*. Simultaneously, the contents of stachydrine, choline and synephrine in these herbs present similar changing trends along with the delay of harvest time. However, the contents of synephrine decrease most rapidly, while those of stachydrine decrease most slowly. Based on these above, comparing with the pharmacological activities and pharmacokinetics reported of stachydrine and synephrine, it indicated that stachydrine and synephrine can be considered as a pair of bioactive equilibrists, especially in the cardio-cerebrovascular protection from these citrus herbs. Simultaneously, it confirmed that stachydrine should play an important role in the pharmacological functions, especially in dual-directionally regulating the uterus and various beneficial effects on cardio-cerebrovascular system, kidney and liver, of these citrus herbs.

Keywords: Choline; *Citrus*; method; analysis; decoction; *Zhishi*; *Zhiqiao*; effect; cardiovascular protection; uterus

1. Introduction

The fruits or peels of some citrus genus plants were traditionally used as *Qi*-regulating Chinese herbs [1]. Among them, herbs *Zhishi* (Aurantii fructus immaturus) and *Zhiqiao* (Aurantii fructus) are respectively collected from the dried young and near-mature fruits of *Citrus aurantium* L., *Citrus sinensis* Osbeck, or their cultivated varieties, and respectively harvested in May to June, and July. Also, these herbs were widely used in the prescriptions for treating various gastrointestinal and cardiovascular diseases in clinic, and many ingredients, including alkaloids, flavonoids, essential oil, coumarins, limonoids, *etc*., were identified and considered to be responsible for their pharmacological functions [2,3].

It was reported that two alkaloids synephrine and *N*-methyltyramine discovered in these herbs can produce various adrenergic effects, including raising blood-pressure, constricting peripheral blood vessels, dilating the pupil, stimulating the uterus, and relaxing the intestines [3-5]. However,

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some animal experiments and clinical results indicated that the decoctions or aqueous extracts of *Zhishi* and *Zhiqiao* probably connoted some pharmacological effects contrary to synephrine and *N*-methyltyramine [6]. For example, (1) transiently or inapparently raising, or sometimes even declining the blood pressure [7,8]; (2) various cardiovascular protections [9,10]; (3) an excitatory effect to the *in vitro* and *in vivo* uteruses of pregnant and nonpregnant rabbits [11]; (4) a two-way regulating effect on gastrointestinal smooth muscle, namely not only exciting the gastrointestinal tract and enhancing its peristalsis, but also reducing the tension of gastrointestinal smooth muscle and relieve spasm [12]. These facts showed that there are probably some other ingredients with cholinergic or anti-cholinergic activities in the decoctions or aqueous extracts of *Zhishi* and *Zhiqiao* although some reports indicated that citrus flavonoids and essential oil should play a certain role in these complicated functions [13-15].

Based on these above, it is easy to associate with the agonists of cholinergic receptor or the antagonist of adrenergic receptor, while more probably the former. The extended structure-activity relationships indicated that whether cholinergic or anticholinergic agents present a similar structural characterization (Figure 1). Namely, as shown on the center of Figure 1, a nitrogen atom of tertiary amine or quaternary ammonium is linked to an oxygen atom by an alkane chain of 2 to 3 carbons, or by a hydrocarbon chain of 3 to 4 carbons containing *cis*- double bonds, with the commensurate space distance. This structural fragment can bind with the cholinergic receptor or choline esterase, and then initiate various cholinergic or anticholinergic activities. This led us to focus on the discovery of choline analogs from these herb decoctions. As choline does not contain any chromophore and has no ultraviolet absorption, it is unable to probe according to our previous structure-oriented thinking using the HPLC method [16]. However, choline has the structural fragment of quaternary ammonium, and belongs to alkaloids. Thereby, here an efficient and specific chromogenic reagent was first explored for establishing a sensitive thin layer chromatography (TLC) method detecting choline analogs from these citrus herbs or other plant resources. Then, probable choline analogs in these herb decoctions were isolated and identified, and the quantitative analyses of them and synephrine were performed for further clarifying the substance bases of various pharmacological activities presented by these citrus Chinese herbs.

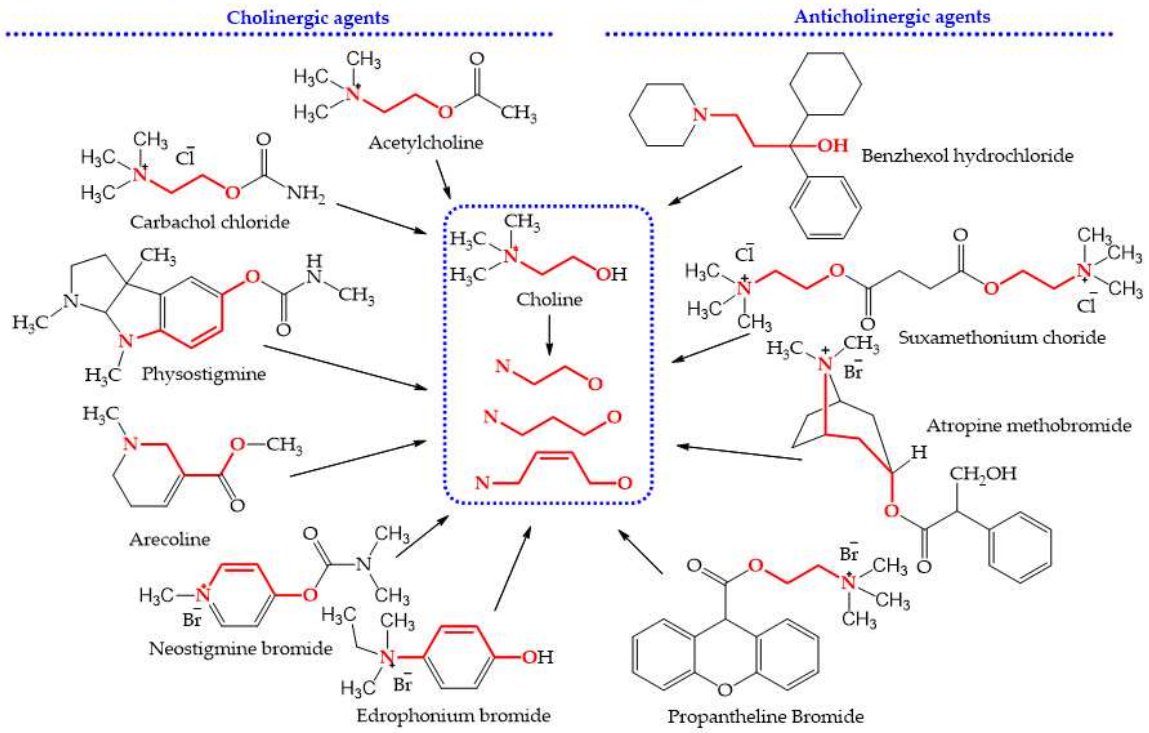

Figure 1. The extended structure-activity relationships of cholinergic and anti-cholinergic agents.

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2. Results

2.2. Detection of Choline Analogs in These Citrus Chinese Herbs

For detecting possible choline analogs from botanical resources, choline chloride was used as a positive indicator. Considering that synephrine, γ-aminobutyric acid and their analogs were already discovered in these four citrus Chinese herbs [2,3], γ-aminobutyric acid and synephrine were taken as two controls to respectively exclude the similar primary and secondary amines in many botanical samples. Various proportions of two solvent systems I (ethyl acetate-95% ethanol-formic acid) and II (*n*-butanol-glacial acetic acid-water) were tested for botanical samples, and the results indicated that there was no obvious difference of developing effects, between these two solvent systems. The better proportions of solvent systems I and II were 10:4:5 and 4:1:5 (lower layer), respectively. Considering that solvent system II developed more slowly on the plate than solvent system I, and simultaneously a tiering operation should be completed for solvent system II, solvent system I with the proportion of 10:4:5 was selected for following TLC analysis.

Furthermore, the color reactions (Figure S1) on the thin layer plates indicated that choline and synephrine respectively presented brown and orange-red strips for improved Dragendorff's reagents, while no strips was observed from all the lanes of choline, synephrine and γ-aminobutyric acid after colored with Dragendorff's and Wagner reagents. Although Dragendorff's and Wagner reagents are two broad-spectrum chromogenic agents for alkaloids, they generally don't work for many compounds with smaller molecular weights, such as both choline and synephrine. From Figure S1, it indicated that the improved Dragendorff's reagent can effectively detect possible choline analogs which usually have smaller molecular weights. Besides, it can also color synephrine, a catecholamine amine with small molecular weight, for an orange red. This further indicated that a compound presented red or orange-red like many alkaloids is unlikely a choline analog after colored with the improved Dragendorff's reagent.

According to the general procedure of TLC analysis, possible choline analogs in four citrus Chinese herbs were detected with a positive indicator of choline, using the optimized developing solvent and chromogenic reagent. The results (Figure 2a) showed that all these four herbs contained choline or a same probable choline analog, while there are probably more analogs and larger contents in the decoction sample of *Zhishi* than those of *Zhiqiao*, *Qingpi* and *Chenpi*. The TLC analyses further indicated that samples from different producing areas contain different choline analogs with different contents, such as the TLC analyses for ten *Zhishi* samples from different producing areas (Figure 2b).

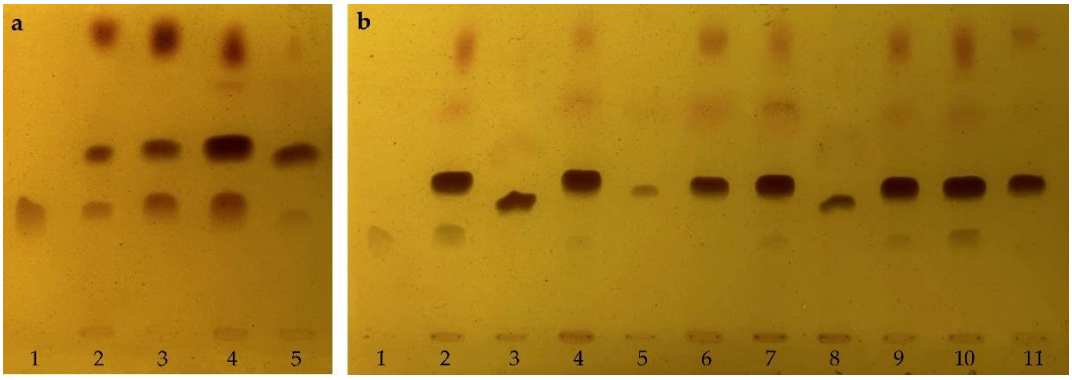

Figure 2. Detection of choline analogs infour Chinese herbs form Citrus genus. **a**: citrus Chinese herbs; 1, Choline; 2, Chenpi (No. 2010019); 3, Qingpi (No. 2010041); 4, Zhishi (No. 2010001); 5, Zhiqiao (No. 2010032). **b**: Chinese herb Zhishi from different producing areas in China; 1, Choline; 2, No. 2008004 (Zizhong, Sichuan); 3, No. 2010002 (Shanggao, Jiangxi); 4, No. 2010003 (Danleng, Sichuan); 5, No. 2010004 (Baisha, Chongqing); 6, No. 2010005 (Anyue, Sichuan); 7, No. 2010006 (Ziyang, Sichuan); 8, No. 2010007 (Tonglian, Sichuan); 9, No. 2010008 (Jintang, Sichuan); 10, No. 2010009 (Jiangjin, Chongqing); 11, No. 2010010 (Lezhi, Sichuan).

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2.3. Isolation and Identification of Choline Analogs in Citrus Chinese Herb Zhishi

Considering that the decoctions of *Zhishi* samples contained more choline analogs and lager content, ten samples of *Zhishi* from different producing areas (Figure 2b) were mixed and pulverized for obtaining more probable analogs of choline. Using the preparative TLC, probable choline analogs **1**, **2** and **3** were isolated from the sampling solution of *Zhishi* according to the process in section "4.2.2.". Based on their spectroscopic data of HRMS, ^{1}H and/or ^{13}C NMR, compounds **1**, **2** and **3** were respectively identified as choline, stachydrine (Figure 3) and synephrine. It indicated that there is a similar fragment, surrounded by a blue coil on Figure 3, in the structure of stachydrine and choline.

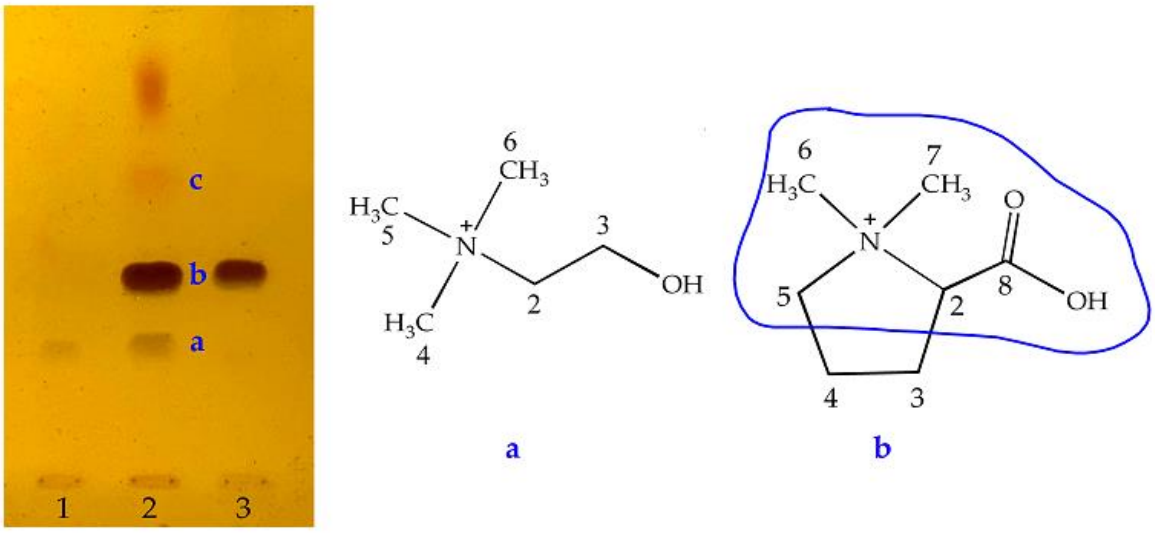

Figure 3. Chemical structure of probable choline analogs. 1, standard choline; 2, the mixed sample of Chinese herb *Zhishi*; 3, standard stachydrine; spots **a**, **b** and **c** correspond to compounds **1**, **2** and **3**, and the chemical structures of choline analogs **1** and **2** were respectively shown as **a** and **b** on the right of the thin layer plate.

The related data for their identification were presented as follows.

1: a white amorphous powder; a brown spot colored with improved Dragendorff's reagents on the thin layer plates; HRESIMS *m/z* 104.1060 $[M]^+$ (calcd. For $C_5H_{14}NO$, 104.1075); ^{1}H NMR (MeOH-d_4, 600 MHz) δ: 3.91 (H-3), 3.56 (H-2) and 3.24 (H-4 to H-6, s); ^{13}C NMR (MeOH-d_4, 150 MHz) δ: 68.9 (C-2), 56.9 (C-3) and 54.5 (C-4, 5 and 6).

2: a white amorphous powder; a reddish-brown spot colored with improved Dragendorff's reagents on the thin layer plates; HRESIMS *m/z* 144.1010 $[M]^+$ (calcd. For $C_7H_{14}NO_2$, 144.1025); ^{1}H NMR (D_2O, 400 MHz) δ: 4.07 (1H, t, J = 9.2 Hz, H-2), 3.70 (1H, m, H-5a), 3.53 (1H, m, H-5b), 3.29 (3H, s, H-6), 3.10 (3H, s, H-7), 2.48 (1H, m, H-3a), 2.33 ~ 2.24 (1H, m, H-3b) and 2.23 ~ 2.11 (2H, m, H-4); ^{13}C NMR (D_2O, 100 MHz) δ: 171.3 (C-8), 76.4 (C-2), 67.2 (C-5), 52.1 (C-6), 45.9 (C-7), 25.3 (C-3) and 18.6 (C-4).

3: a white amorphous powder; an orange-red spot colored with improved Dragendorff's reagents on the thin layer plates; HRESIMS *m/z* 168.1009 $[M+H]^+$ (calcd. for $C_9H_{14}NO_2$, 168.1024), 150.0903 $[M+H-H_2O]^+$ (calcd. For $C_9H_{12}NO$, 150.0919) which presented as base peak ion.

The above indicated that compound **3** is not a choline analog. After spraying with improved Dragendorff's reagent, the spots of compounds **1** and **2**, as choline analogs, presented brown or reddish-brown color, while compound **3** showed orange-red spot on the thin layer plate. These indicated that the compounds represented by the spots are unlikely choline analogs if the color of spots is red. The detailed discussion would be involved in section "3. Discussion".

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2.4. The Contents of Stachydrine, Choline and Synephrine in Four Citrus Chinese Herbs

Along with the identification of stachydrine and choline from four citrus Chinese herbs Zhishi, Zhiqiao, Qingpi and Chenpi, so far it was known that three alkaloids as stachydrine, choline and synephrine are extensively distributed in these herbs, while their proportions and contents in these herbs were different from each other. Considering that these herbs have different pharmacological functions according to the theory of Chinese medicine, it was worth exploring whether there were some relationships between the pharmacological functions of these herbs and the proportions and contents of these three ingredients. To achieve this, the contents of these three ingredients were determined.

2.4.1. Validation of Quantitative Analyses

As there is no chromophore in the structures of stachydrine and choline, it is unsuitable for their quantitative analyses using HPLC-UV method. Based on the detection procedure of choline analogs and their separation effects on thin layer plates, the TLCS method was prior to be considered for the quantitative analyses of of stachydrine and choline. However, the quantitative analysis for synephrine was established using the HPLC-UV method, refering to that descibed in Chinese pharmacopoeia [1].

The methodology validation showed that the chromatographic peaks of both choline and stachydrine presented good symmetry, and both two compounds can be well separated with an identical resolution of 1.26 from their nearest peaks, according to the procedure of TLCS analysis descirbed in subsection "4.4.1.". The limit of detection (LOD) and limit of quantitation (LOQ) for stachydrine were respectively 1.0 μg (0.20 mg·mL^{-1}) with a relative standard deviation (RSD) of 0.08% and 2.0 μg (0.40 mg·mL^{-1}) with a RSD of 0.18%, and those for choline were respectively 0.5 μg (0.10 mg·mL^{-1}) with a RSD of 0.08% and 0.8 μg (0.15 mg·mL^{-1}) with a RSD of 0.18%. The repeatability tests showed that the RSDs for a strip of stachydrine and choline on a thin layer plate were 0.34% and 0.48%, respectively. The precision tests indicated that the RSDs for six strips of stachydrine and choline on the same thin layer plate were respectively 1.20% and 3.55%, and those for the identical solutions of stachydrine and choline on six thin layer plates were respectively 4.05% and 4.15%. A good linearity correlation $y = 6565.7x + 5522.7$ ($r = 0.9996$) (Figure S2a) between the amounts (x) and peak areas (y) was presented for stachydrine in a range from 1.0 to 14.0 μg, and an acceptable linearity correlation $y = 12302.1x - 909.3$ ($r = 0.9987$) (Figure S2b) was presented for choline in a range from 1.0 to 4.0 μg. Using a powder sample of Zhishi (No. 2010001), the reproducibility was assessed, and the results showed that the contents of stachydrine and choline in this sample were 0.302% (with a RSD value of 2.39%) and 0.085% (with a RSD value of 2.03%), respectively. Using this Zhishi sample, the recoveries of choline and stachydrine were respevtively tested. The results showed that the average recovery of stachydrine was 100.28% with a RSD value of 3.08 (Table S1), and that of choline was 101.04% with a RSD value of 2.31 (Table S2). These above together indicated that the established TLCS methods were valid, and can be used for the quantitative analyses of stachydrine and choline in these herbs.

Similarly, the chromatographic peaks of synephrine presented good symmetry, and can be well separated with a resolution of 4.06 from its nearest peaks, according to the procedure of HPLC analysis described in subsection "4.5.1.". The limit of detection (LOD) and limit of quantitation (LOQ) for synephrine were respectively 0.02 μg and 0.05 μg. The repeatability experiment presented a RSD value of 0.42% for a standard solution of synephrine. A good linearity correlation $y = 4435448.7x + 2217.2$ ($r = 1.0000$) (Figure S2c) between the amounts (x) and peak areas (y) was presented for synephrine in a range from 0.5 to 32.0 μg. Using a powder sample of Zhishi (No. 2010001), the reproducibility experiment showed that the contents of synephrine in this sample were 1.10% with a RSD value of 1.77%, and the sample solution showed a good stability with a RSD value of 1.60% in 24 h. Using this Zhishi sample, the recovery of synephrine was tested, and the results showed that the average recovery of synephrine was 99.89% with a RSD value of 3.19 (Table S3). These above together indicated that the established HPLC method were valid, and can be used for the quantitative analyses of synephrine in these herbs.

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2.4.2. Contents of Stachydrine, Choline and Synephrine in Four Citrus Chinese Herbs

Using above valid methods for the quantitative analyses, the contents of stachydrine, choline and synephrine in these citrus herbs were performed, and the results were shown in Table 1.

Table 1. Contents of stachydrine, choline and synephrine in four Chinese herbs from Citrus genus. [a]

Name of Chinese herbs	Producing Area	Batch No.	Harvest dates	Contents in chinese herb ($mg \cdot g^{-1}$)			Content ranges ($mg \cdot g^{-1}$)		
				Stachydrine (SC)	Choline (CL)	Synephrine (SN)	Stachydrine (SC)	Choline (CL)	Synephrine (SN)
Zhishi	Zizhong, Sichuan	2010001	June, 2020	3.10	0.87	11.03	2.94 ~ 8.53	0.00 ~ 0.87	3.56 ~ 21.07
	Shanggao, Jiangxi	2010002	June, 2020	6.42	0.16	21.07			
	Danleng, Sichuan	2010003	June, 2020	5.35	0.39	3.56			
	Baisha, Chongqing	2010004	June, 2020	3.23	— [b]	5.21			
	Anyue, Sichuan	2010005	June, 2020	5.31	0.42	19.84			
	Ziyang, Sichuan	2010006	June, 2020	6.46	0.47	20.10			
	Tongliang, Chongqing	2010007	June, 2020	3.74	0.11	4.34			
	Jintang, Sichuan	2010008	June, 2020	8.53	0.29	12.58			
	Jiasi, Chongqing	2010009	June, 2020	2.94	0.21	9.64			
	Lezhi, Sichuan	2010010	June, 2020	6.48	0.31	10.80			
Zhiqiao	Baisha, Chongqing	2010031	July, 2020	1.43	0.10	2.42	1.43 ~ 5.13	0.00 ~ 0.21	0.00 ~ 2.42
	Tongnan, Chongqing	2010032	July, 2020	2.51	0.21	1.82			
	Bazhong, Sichuan	2010033	July, 2020	5.13	0.14	0.59			
	Zizhong, Sichuan	2010034	July, 2020	3.92	0.20	1.25			
	Jiasi, Chongqing	2010035	July, 2020	3.11	0.12	1.77			
	Zhangshu, Jiangxi	2010036	July, 2020	2.31	0.19	1.73			
	Dazu, Chongqing	2010037	July, 2020	2.66	0.19	1.63			
	Dazhu, Sichuan	2010038	July, 2020	4.68	—	—			
	Ji'an, Jiangxi	2010039	July, 2020	1.62	—	—			
Qingpi	Quzhou, Zhejiang	2010041	July, 2020	2.35	0.34	5.16	0.99 ~ 3.51	0.21 ~ 0.60	4.39 ~ 7.19
	Zhangshu, Jiangxi	2010042	July, 2020	2.63	0.32	6.18			
	Danleng, Sichuan	2010043	July, 2020	2.22	0.47	6.11			
	Fengyuzhen, Sichuan	2010044	July, 2020	1.93	0.32	6.37			
	Ziyang, Sichuan	2010045	July, 2020	0.99	0.29	5.30			
	Huangshui, Sichuan	2010046	July, 2020	1.15	0.21	6.29			
	Meishan, Sichaun	2010047	July, 2020	3.51	0.47	5.08			
	Pingshan, Sichuan	2010048	July, 2020	1.64	0.60	7.19			
	Ji'an, Jiangxi	2010049	July, 2020	0.99	0.59	4.39			
	Shuangliu, Sichuan	2010050	July, 2020	2.36	0.53	5.56			
Chenpi	Jintang, Sichuan	2010011	January, 2020	0.96	—	1.98	0. 86 ~ 3.26	0.00 ~ 0.20	1.86 ~ 3.80
	Yibin, Sichuan	2010012	January, 2020	1.03	—	2.04			
	Nanchong, Sichuan	2010013	January, 2020	1.92	0.16	2.71			
	Meishan, Sichuan	2010014	January, 2020	2.93	—	1.86			

Neijiang, Sichuan	2010015	January, 2020	0.86	0.12	2.74
Yiyang, Hunan	2010016	January, 2020	3.26	—	3.80
Anyue, Sichuan	2010017	January, 2020	1.33	0.18	2.43
Lezhi, Sichuan	2010018	January, 2020	1.16	0.09	2.16
Dazhou, Sichuan	2010019	January, 2020	1.89	0.20	2.52
Xinhui, Guangzhou	2010020	January, 2020	1.43	—	2.74

[a]: These citrus herbs were commercially available from Chengdu Huichu Technology Co., Ltd. in China; herbs Zhishi and Zhiqiao are respectively the dried young and near-mature friuts of Citrus aurantium L. or its cultivated varieties, and herbs Qingpi and Chenpi are the dried peel respectively from young (or immature) and mature friuts Citrus reticulata Blanco or its cultivated varieties. [b]: —, the content was lower than the LOD and was set as 0.00 for following calculations.

From Table 1, the contents of three ingredients in four herbs fluctuate greatly. Overall, the contents of stachydrine and synephrine are obviously larger than those of choline, and the content fluctuations of synephrine are greater than those of stachydrine in these herbs. Simultaneously, the contents of synephrine and stachydrine is obviously larger in Zhishi than in other three herbs. It is worth noting that the contents of synephrine in herb Zhiqiao is the smallest in these herbs, while those of stachydrine in herb Zhiqiao is very large and just lower than in herb Zhishi.

2.4.3. Statistical Analysis for the Content Data of Three Ingredients

To make the content differences of these three ingredients clearer for trying to explain the differences in pharmacological functions of these herbs, the data in Table 1 were further analyzed using statistical methods. The results confirmed that the contents of stachydrine and synephrine are larger in herb Zhishi than in other three berbs ($P < 0.05$ or $P < 0.01$), and while those of choline are larger in Qingpi (or/and Zhishi) than Zhiqiao and Chenpi ($P < 0.05$ or $P < 0.01$). Simultaneously, stachydrine is the largest one of these three ingredients in Zhiqiao ($P < 0.05$ or $P < 0.01$).

Since herbs Zhishi and Qingpi are respectively the dried young friuts of Citrus aurantium L. (or its cultivated varieties) and Qingpi and Chenpi are the dried peel from young (or immature) friuts Citrus reticulata Blanco (or its cultivated varieties), it was further concluded that all these three ingredients will reduce ($P < 0.05$) along with the prolongation of growth time for Citrus genus plants except for stachydrine in herbs Qingpi and Chenpi. Among them, the contents of synephrine decrease most rapidly, and while those of stachydrine decrease most slowly. Although all their contents reduce along with the prolongation of growth time for these Citrus genus plants, the contents of synephrine are significantly larger than those of stachydrine and choline except for that those of stachydrine are largest in herb Zhiqiao. Moreover, depending on their descending speeds and degrees of these three components in the fruits and pericarps along with the growth time of these Citrus genus plants , it was further inferred that synephrine and stachydrine distributed more in exocarp than in mesocarp, and while there is possibly no obvious distribution difference of choline in exocarp and mesocarp, since herbs Zhishi (or Zhiqiao) and Qingpi (or Chenpi) are respectively derived from the fruits and the pericarps. This inference was in accordance with a previous report [17].

2.5. Comprehensive Analyses for the Pharmacological Effects of Stachydrine and Synephrine

These four herbs derived from the fruits or peels of *Citrus* genus plants. Herbs *Zhishi* and *Zhiqiao* can be respectively originated from the fruits of *Citrus aurantium* L. or its cultured varieties at different harvested times, and while herbs *Qingpi* and *Chenpi* can be respectively originated from the peels of *Citrus reticulata* Blanco or its cultured varieties at different harvested times [1]. Their decoctions or

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water extracts have various pharmacological effects on the digestive system, cardiovascular system, respiratory system, and so on [2,3,11]. Many reports indicated that flavonoids, alkaloids, coumarins, essential oil and limonoids are main components in these herbs [2,3]. It was reported that some flavonoids (such as narirutin, naringin, hesperidin, neohesperidin and nobiletin) and alkaloids (such as synephrine and *N*-methyl tyramine), with a higher content and a wider distribution in these herbs, have various bioactivities to the digestive system, cardiovascular system and respiratory system, and which are mainly responsible for their pharmacological effects of these herbs [11,12,18]. However, some pharmacological activities of these herbs to human body have little related investigations, such as an excitatory effect on the *in vitro* and *in vivo* uteruses of both pregnant and non-pregnant rabbits for herbs *Zhishi*, *Zhiqiao* and *Qingpi*. Moreover, some related investigations remain insufficiently clear, such as which components are responsible for the two-way regulating effects on gastrointestinal smooth muscle, showed by the decoction of *Zhiqiao* [12,18].

Considering that the decoctions or aqueous extracts of these herbs conceal some possible cholinergic activities, here probable choline analogs in these herbs were detected and determined. The results from Table 2 showed that stachydrine (or plus choline) and synephrine presented commensurate contents in these herbs and similar changing trends along with the increase of growth time. However, the contents of synephrine decrease most rapidly, while those of stachydrine decrease most slowly. Since synephrine is a sympathomimetic amine and has intrinsic sympathomimetic activity, it was inferred that they possibly have different or even contrary pharmacological activities. If this is true, different contents and proportions of both two compounds in these herbs would have important impacts on their whole pharmacological functions on human body, and which would probably give some reasonable interpretations for the difference in pharmacological functions of these herbs, although other components also have important roles. Moreover, this would also fluctuate the pharmacological effects of these herbs with different producing areas and harvested times. To clarify these, here main bioactivities of stachydrine (plus choline) and synephrine were summarized and listed in Table 3.

Table 2. Statistical analyses for the contents of stachydrine, choline and synephrine in four citrus herbs. [a]

Name of Chinese herbs	Average content ± SD (mg/g) [b]			Sequencing of the contents of SC, CL and SN [c]	Sequencing of the contents of SN, and SC plus SC [d]
	Stachydrine (SC)	Choline (CL)	Synephrine （SN）		
Zhishi	5.16 ± 1.87$^{**\#\#++}$	0.32 ± 0.24$^{*\#}$	11.82 ± 6.61$^{\#\#+}$	SN$^{!!}$ > SC > CL$^{!!}$	SN > (SC + CL)‡‡
Zhiqiao	3.04 ± 1.29$^{\#+}$	0.13 ± 0.08^{++}	1.25 ± 0.86$^{\#\#++}$	SC§ > SN > CL§§	(SC + CL)‡ > SN
Qingpi	1.98 ± 0.81*	0.41 ± 0.14$^{**\#\#}$	5.76 ± 0.81$^{**\#\#}$	SN$^{!}$ > SC > CL$^{!!}$	SN > (SC + CL)‡‡
Chenpi	1.68 ± 0.83*	0.08 ± 0.08^{++}	2.50 ± 0.56^{++}	SN$^{!!}$ > SC > CL$^{!!}$	SN > (SC + CL)‡
Sequencing in herbs	*Zhishi* > *Zhiqiao* > *Qingpi* (*Chenpi*)	*Qingpi* (*Zhishi*) > *Zhiqiao* (*Chenpi*)	*Zhishi* > *Qingpi* > *Chenpi* > *Zhiqiao*		

[a]: The data before analysis were shown in Table 1. [b]: *, $^{+}$ and $^{\#}$ indicated that the differences are significant ($P < 0.05$) respectively compared with those of Zhiqiao, Qingpi and Chenpi; **, $^{++}$ and $^{\#\#}$ indicated that the difference are significant ($P < 0.01$) respectively compared with those of Zhiqiao, Qingpi and Chenpi. [c]: $^{!}$ and § indicated that the difference are significant ($P<0.05$) respectively compared with those of stachydrine (SC) and synephrine (SN); $^{!!}$ and §§ indicated that the difference are significant ($P < 0.01$) respectively compared with those of stachydrine (SC) and synephrine (SN). [d]: ‡ and ‡‡ indicated that the difference are significant ($P < 0.05$) or remarkably significant ($P < 0.01$) compared with those of synephrine (SN).

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Table 3. Main bioactivities of stachydrine (plus choline) and synephrine [a.]

Effected tissues, organs or systems	Pharmacological effects	
	Synephrine	Stachydrine (Choline)
Eye	Exciting α_1-adrenorecepor and dilating the pupil [19].	/
cardio-cerebrovascular system	A partial agonist of α_1-adrenoreceptor and an antagonist of α_2-adrenoreceptor, and can weakly bind on α_1- and α_2-adrenoreceptors. The effects on β_1- and β_2-adrenoreceptors are very small and can be ignored [4,5,10,20-22]. 1) Constricting peripheral blood vessels including mesenteric artery, and raising blood pressure; 2) Complex responses of the coronary artery by the excitation of α_1-adrenoceptor and TAARs [20]; 3) Constricting aorta directly by the excitation of α_1-adrenoceptor and 5-HT1D [23], not by 5-HT1B and β-receptor [21]; 4) Cerebral vasoconstriction deduced from that it acts on the α_1-adrenoceptor.	Cardiovascular system protection [24]: 1) Accelerating blood circulation, increasing coronary and myocardial blood flow in **adrenaline-induced** myocardial ischemia [25,26]; 2) Relieving myocardial necrosis, lowering blood viscosity and vascular resistance, improving microcirculation [25,26]; 3) Slowing heart rate and decreasing cardiac output [25,26]; 4) Suppressing and ameliorating myocardial fibrosis [27,28]; 5) Ameliorating **isoproterenol-induced** cardiac hypertrophy and fibrosis [29]; 6) Inhibiting **norepinephrine-induced** cardiomyocyte hypertrophy [30-32]; 7) Rapid vascular relaxation mediated by the activation of endothelial nitric oxide synthase in vascular endothelial cells [33]; 8) Ameliorating **endothelial dysfunction induced by homocysteine** [34].
Blood	Increasing the level of platelet [35].	Inhibiting platelet aggregation and ameliorating platelet-mediated thrombo-inflammation [25,26,36];
Neuroprotective effects	/	1) Protecting the neuronal injury [37]; 2) Inhibiting inflammatory reactions and improving pathological changes after cerebral ischemia [38]; 3) Inhibition of neuronal apoptosis, improvement of energy metabolism disorder, and microcirculation of brain [39].
Respiratory system	No bronchial constriction [40].	Antitussive effects by reducing citric acid–induced coughing [41].
Digestive system	A partial agonist of α_1-adrenoreceptor and an antagonist of α_2-adrenoreceptor. 1) Relaxing the intestinal smooth muscle and the intestine [3]; 2) A modest reduction in contractions for rabbit duodenum [40]; 3) Both of above are also supported with it is an antagonist of α_2-adrenoreceptor [5].	1) Treating non-alcoholic fatty liver disease [24]; 2) Ameliorating carbon tetrachloride-induced hepatic fibrosis [42]; 3) For choline, maintaining the function and health of liver [43,44].
Uterus	Uterine contraction (pregnancy), deduced from the fact that synephrine is an agonist α_1-adrenoreceptor [45].	Regulation uterus effect (pregnancy and non-pregnancy) [25,46]: 1) Stimulation of uterine contraction [47,48]; 2) Inhibition of convulsive uterus [49]; 3) Reducing uterine bleeding [50].
Blood sugar	Inhibiting α_1-adrenoreceptor and α-glycosidase, and presenting hypoglycemic effect which can be also deduced from that it is an antagonist of α_2-adrenoreceptor [5,51,52].	Ameliorating and protecting high-glucose induced endothelial cell senescence by upregulation of SIRT1 and downregulation of $p16^{INK4A}$ [53].
Anti-inflammatory effect	/	1) Inhibition of TXB2 and IL-10 secretion, and production of NO [54]; 2) Inhibition of NF-κB and AKT signal pathways [55]; 3) Improvement of cellular membrane permeability, and inhibition of inflammatory factors and lipid peroxidation [56].
Antidepressant activity	Anti-depressant activity by modulating noradrenergic neurotransmission and stimulating α_1-adrenoceptor [57-59].	/
Anti-obesity	Weight loss, anti-obesity, and regulating fat metabolism, due to that synephrine is a partial agonist β_3-adrenoreceptor, and can weakly bind on β_3-adrenoreceptor [60], together with lipolytic and thermogenic effects [61].	/
Renal protection	/	1) Reducing and ameliorating renal interstitial fibrosis [62]; 2) Ameliorating hydrogen peroxide-induced renal tubular epithelial cell injury [63]; 3) Protecting adenine-induced chronic renal failure [64];

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Pharmacokinetics	Pharmacokinetics character [65,66,67]: 1) Oral ingestion absorption was fast, and the time to peak is approximately ranged from 1 to 2 h after administration; 2) The biological half-life is about 2 h; 3) The bioavailability is approximately 22%; 4) The metabolism is exerted predominantly in the liver, and it can be rapidly removed from the bloodstream by hepatic uptake; 5) Cannot cross the blood-brain barrier	Pharmacokinetics character [68,69]: 1) Rapid absorption after oral administration 2) Fast and extensive distribution; 3) The biological half-life is about 4 h; 4) The time to peak is approximately 3 h after administration; 5) The bioavailability is above 90%; 6) Most excreted from urine.

[a]: /, no related reports obtained.

From Table 3, synephrine, as a partial agonist of α_1-adrenoreceptor and an antagonist of α_2-adrenoreceptor, can constrict peripheral blood vessels, cerebrovascular and aorta, while stachydrine, an ingredient coexisting with synephrine in these citrus herbs, present various cardio-cerebrovascular protections including rapid vascular relaxation, accelerating blood circulation, increasing coronary and myocardial blood flow, relieving myocardial necrosis, slowing heart rate and decreasing cardiac output, suppressing and ameliorating myocardial fibrosis, ameliorating cardiac hypertrophy and fibrosis, especially some results were obtained from animal models induced by adrenergic receptor agonists (marked as bold front in Table 3). Simultaneously, synephrine would increase the level of platelet [35], while stachydrine can inhibit platelet aggregation and ameliorate platelet-mediated thrombo-inflammation [25,26,36]. Synephrine can contract uterus (pregnancy) [45], while stachydrine can regulate uterus, such as inhibition of convulsive uterus [94], stimulation of uterine contraction [47], and reducing uterine bleeding [50]. Moreover, synephrine can be rapidly absorbed and predominantly metabolized in the liver [70], and this would lead to bring out some possible unfavorable influences on the liver especially at large doses for citrus herbs or some related juices contained synephrine. However, stachydrine can rapidly relax blood vessels by acting the endothelial nitric oxide synthase in vascular endothelial [33], and has various helpful effects for the liver, such as anti-inflammatory action, ameliorating hepatic fibrosis [42] and treating non-alcoholic fatty liver [24]. Thereby, the unfavorable influence on liver from synephrine would be theoretically eradicated by stachydrine coexisting in these citrus herbs.

From the pharmacokinetics characters (Table 3) of synephrine and stachydrine, both compounds can be rapidly absorbed after oral administration, while the relative bioavailability is approximately 22% for synephrine and 90% for stachydrine, respectively. Considering that the contents of stachydrine in these herbs except *Zhiqiao* is approximately 1/3 to 2/3 of synephrine, some pharmacological contributions from the bioavailable differences of both two ingredients would be balanced each other, to a great extent, by their contents in these herbs. Together with most contrary pharmacological activities mentioned above (shown in Table 3), these indicated that both ingredients can be considered, only from their pharmacological activities, as a pair of antagonists in these citrus herbs. However, both of them not only present many contrary bioactivities and also show some different or synergetic bioactivities, such as neuroprotective, hepatoprotective, renal protective, antitussive and anti-inflammatory effects from stachydrine, and gastrointestinal relaxation, antidepressant and anti-obesity activities from synephrine, and their synergistic activities in uterine contraction and anti-diabetes. Thereout, when they are used for other medicinal purposes, their contrary bioactivities usually acting as their individual adverse effects can be partly cancelled by each other. From this view, they can be also considered as a pair of synergists or associates from their contributions to the pharmacological functions and safety of these herbs.

It is noteworthy that sometimes there are no related reports for another compound when some pharmacological activities are presented in Table 3 for one of synephrine and stachydrine. However, this does not mean that it has no similar, different or even contrary bioactivities to the identical organ or tissue. As above described, the stachydrine was eventually identified from these herbs, using the detection method of choline analogs. Simultaneously, from the pharmacological activities especially various cardio-cerebrovascular effects, stachydrine presents various contrary effects compared to synephrine and other adrenergic receptor agonists in Table 3 (marked in bold front at items 1), 5), 6)

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and 8) of row "cardio-cerebrovascular system"). Thereby, it indicated that stachydrine seems to have some cholinergic activities, and more like an agonist of M-type cholinergic receptor. Considering that the contents changed with the harvest time of these herbs are simultaneous and similar for stachydrine and synephrine, stachydrine and synephrine can be also considered as a pair of bioactive equilibrists in *Citrus* genus like a pair of sympathetic and parasympathetic neurotransmitters in human body.

Based on these above, many confused facts for the aqueous extract or the decoction of *Zhishi*, *Zhiqiao*, *Qingpi* or/and *Chenpi*, including an excitatory effect on the *in vitro* and *in vivo* uteruses of both pregnant and non-pregnant rabbits, reducing cerebrovascular resistance and increasing cerebral blood flow, constricting gallbladder, and a two-way regulating effect on gastrointestinal smooth muscle, can be scientifically and rationally interpreted. Moreover, some explanations for that four Chinese herbs harvesting from the different parts and growth times of *Citrus* genus have different functions would be also put forward. These will be presented in section "3. *Discussion*".

3. Discussion

Inspired by the traditional and modern pharmacodynamics of four citrus Chinese herbs, here possible choline analogs were discovered from these herbs using the TLC method with a specific chromogenic reagent, and which led to the identification of stachydrine and choline based on their NMR and HRMS data. After this, a TLCS method was first established for the quantitatively analyses of stachydrine and choline, and the contents of both two ingredients and synephrine in 39 samples were determined. Based on this, statistical analysis for the contents of these three ingredients were further performed, and then the pharmacological effects and pharmacokinetics reported of stachydrine and synephrine were comprehensively compared and analyzed. The results showed that stachydrine and synephrine can be considered as a pair of bioactive equilibrists especially in the cardio-cerebrovascular protection from these citrus herbs, and which can, to a great extent, interpret some pharmacological confusions of these herbs. Moreover, some important and relevant aspects would be further discussed and developed as follows.

3.1. A simple Method Detecting Choline Analogs from Plant Resource

Based on the extensive structure-activity relationships of cholinergic and anti-cholinergic agents (Figure 1), choline analogs were speculated to agonists or antagonists of cholinergic receptor, and have various bioactivities to cardio-cerebrovascular, digestive and nervous systems, and eye, *etc*. Simultaneously, some of them also belong betaine analogs, and these compounds have various bioactivities including anti-ulcer, regulating gastrointestinal function and treating liver diseases, especially multiple effects on homocysteine metabolism which is very helpful for the protection to cardio-cerebrovascular and kidney [71]. For example, here stachydrine, also isolated from another Chinese herb *Yimucao* (the overground part of *Leonurus japonicus* Houtt.) [24], was recently reported to have very extensive bioactivities (Table 3). Based on the above TLC procedure discovering stachydrine and choline from these citrus herbs, a simple and rapid method was established for quickly discovering possible choline analogs from botanical resource, and schemed as Figure 4.

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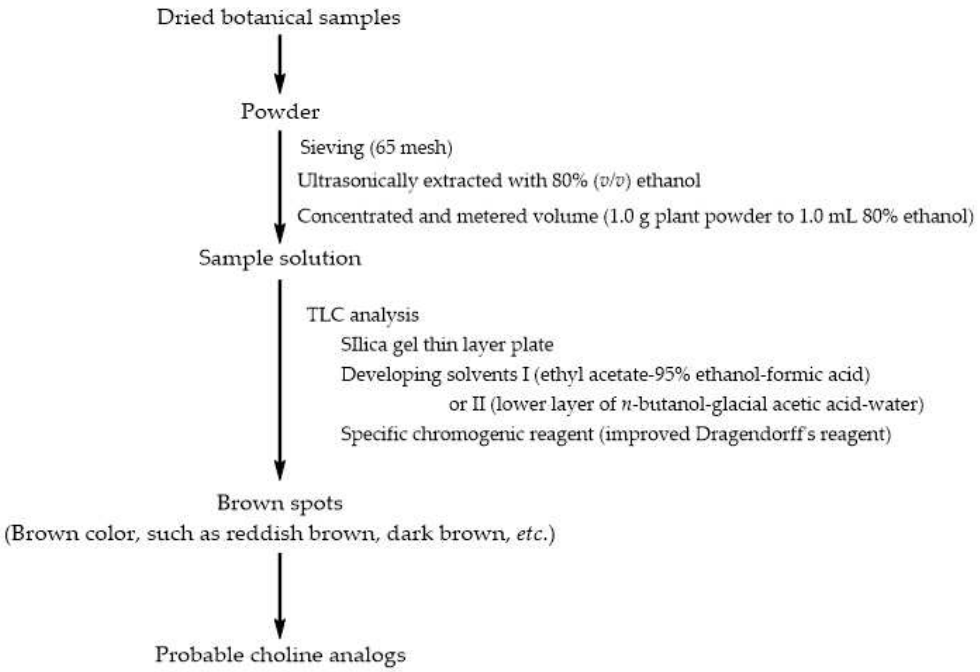

Figure 4. A simple procedure for quickly discovering choline analogs from botanical resources. The specific chromogenic reagent (improved Dragendorff's reagent) was prepared by adding the mixture of bismuth nitrite 0.82 g, potassium iodide 11.06 g and 50% (*v*/*v*) phosphoric acid 90 mL into a 100-mL volumetric flask to make a constant volume using water. .

The procedure (Figure 4) can be used for the discoveries of choline analogs including some betaine analogs. There are two key factors for discovering choline analogs, one is the specific chromogenic reagent (improved Dragendorff's reagent), and another is the color of spots. Generally, two chromogenic reagents Dragendorff's and Wagner were used for the color reaction of most alkaloids, while they present poor effects for some alkaloids with small molecular weight. Considering acidic environment should be provided for the color reaction, the acetic acid was replaced as phosphoric acid in Dragendorff's reagent for improving the chromogenic sensitivity, referring what Zhang N, *et al*. reported [72]. Moreover, if the color of spots is red, such as orange red or brownish red, the compounds represented by the spots are likely not choline analogs, while are likely alkaloids, possibly some polymethoxy flavonoids, or polymethoxybenzenes (such as α- and β-asarones) since the potassium bismuth iodide reagent colors these compounds to orange red or brownish red. This was also supported with the fact that compound **3** was found not a choline analogue, and colored an orange-red spot **c** (Figure 3). Furthermore, the brown color can be more accurately defined with the absorption wavelengths ranged from 550 to 580 nm when the thin layer plate was scanned in 20 to 50 min after taken from the chromogenic reagent.

Moreover, possible choline analogs can be quickly sure by the combinational method of preparative TLC and ^{1}H NMR, since these compounds would present one to three single peaks with some specific ^{1}H chemical shifts ranging from 3.20 to 3.60 ppm (4.45 to 4.75 ppm for methylpyridine type choline analogs, such as trigonelline), assigned to three to nine hydrogens (one to three methyl groups). It is noteworthy that these analyses should eliminate the signal peaks from the deuterated methanol used for the NMR experiments or the possible residual methanol in the process of sample preparation. Thereby, the deuterated solvents MeOH-d_4 should be avoided to use for the NMR experiments as far as possible. Considering these compounds contain a structural fragment of the quaternary ammonium, D_2O is considered as the preferred solvent.

According to the LODs of stachydrine and choline, the detectable content of choline analogs in botanical resoures is approximately from 1.0 to 1.4 μM per gram sample powder, and equal to the content of 0.01% to 0.05% in dried botanical resoures, assuming the molecular weights of choline analogs are less than 500. If high efficient thin layer plate is used for the method, the detection

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sensitivity would be increased. Using this detection procedure of choline analogs, choline and stachydrine were also detected in samples from herbs *Xiangyuan* (Dried fruits of *Citrus wilsonii* Tanaka) and *Foushou* (Dried fruits of *Citrus medica* L. var. *sarcodactylis* Swingle) (Figure S3a), and this was also in accordance with previous publications [41,73]. These indicated that choline and stachydrine are widely distributed in Chinese herbs from *Citrus* genus. Moreover, the results showed that stachydrine and/or choline were also discovered in the leaves of *Citrus* genus plants, with most higher contents than in their individual fruits (Figure S3b). Moreover, some possible choline analogs can be also detected in other botanical resources including some Chinese herbs, such as *Huangliang* (Coptidis Rhizoma), *Juhua* (Dried capitulum of *Chrysanthemum morifolium* Ramat.) and *Chuanxiong* (Dried rhizome of *Ligusticum chuanxiong* Hort.) (Figure S4). This indicated that the method can effectively detect choline or its analogs in various botanical resources.

3.2. The Contents of Stachydrine, Choline and Synephrine in These Citrus Herbs

According to the theory of Chinese medicine, these four citrus herbs have different pharmacological functions [1]. To clarify their substance bases and the reasons of their different functions, many researches on their active ingredients such as flavonoids, alkaloids and essential oil were reported [2,3,74]. Citrus flavonoids and alkaloids were generally considered as two main components responsible for the pharmacological functions of these herbs [74]. However, it remains many confusions that all the pharmacological activities of the ingredients reported in these herbs were difficult to clarify the pharmacological functions of their decoctions. Among them synephrine acts as an agonist of adrenoreceptor, and is widely distributed in these herbs. Here stachydrine and choline were also discovered from these herbs, and were reported to have various bioactivities. Considering that stachydrine and synephrine can be considered as a pair of bioactive equilibrists, it was expected to give some reasonable explanations for above confusions. Thereby, the contents of these three ingredients in these herbs were further determined.

As there is no chromophore in the structures of stachydrine and choline, it is unsuitable for their quantitative analyses using HPLC-UV method although which was also used for the quantitative analyses of stachydrine in Yimucao and choline in various plants. According to the detection procedure of choline analogs, stachydrine and choline can be perfectly isolated from their adjacent spots in these herbs (Figure 1). Thereby, here the TLCS method was prior to select for the quantitative analyses of stachydrine and choline although the HPLC-MS/MS method can be also used for all these three components [69].

The quantitative analyses indicated these three ingredients have similar changing trends along with the increase of growth time. The contents of all three compounds decrease from *Zhishi* (harvested in June) to *Zhiqiao* (harvested in July), and from *Qingpi* (harvested in July) to *Chenpi* (harvested in January of next year). However, the decreasing speed of stachydrine is slower than that of synephrine, and which was also supported by the depth analyses for the ratio values of stachydrine and synephrine, comparing *Zhishi* (0.57 ± 0.37) with *Zhiqiao* (2.65 ± 2.77) ($P < 0.05$), and *Qingpi* (0.35 ± 0.15) with *Chenpi* (0.68 ± 0.33) ($P < 0.05$). Although they were collected from different plants, the changing trends of synephrine, choline and stachydrine in 39 batches of samples are sure according to statistical analyses (Table 2), and among them those of synephrine in various citrus herbs were also supported by many reported [74].

It is noteworthy that some multi-methoxy flavonoids having various bioactivities to cardiovascular, such as nobiletin and tangeretin [75], are reported to be widely distributed in the fruits or peels of many *Citrus* genus plants including these herbs [76,77]. Simultaneously, their contents in the peels are much larger than those in other tissues (such as sarcocarp and seed) of these herbs [76,78], and was in accordance with the distribution of stachydrine in the fruits and peels of these herbs. Moreover, their contents in the fruits or peels of *Citrus* genus plants present decreasing trend along with the delay of harvest time [79,80], and which is just similar to the changing trend of stachydrine and choline. It was reported that stachydrine is a proline betaine, and choline is also a precursor of glycine betaine [71,81,82]. All the bio-syntheses of stachydrine, choline and multi-methoxy flavonoids were catalyzed by *S*-adenosyl-methionine (SAM)-dependent methyltransferases

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with a universal methyl donor SAM [83-85]. Differently, the sub-classified *N*-methyltransferases is responsible for the bio-syntheses of stachydrine and choline, while the sub-classified *O*-methyltransferases is responsible for those of multi-methoxy flavonoids [85]. The similar changing trends of stachydrine/choline and multi-methoxy flavonoids in the same tissue of the *Citrus* genus plants indicated there was a kind of intrinsic mechanism for simultaneously regulating both two sub-classified enzymes with a similar effect, and more probably there are some physiological needs regulating the whole biosynthesis pathway involving SAM-dependent methyltransferases down in the fruits and peels of these citrus genus plants. Thereby, it is further worth studying on the physiological regulations of these *Citrus* genus plants to these components in their fruits and peels, as which would be very helpful for clarifying the different pharmacological functions of these herbs, and the regulation relationship of both two sub-classified enzymes in *Citrus* genus plants.

3.3. Communication between Active Ingredients and Pharmacological Effects of These Herb

It was reported that these citrus herbs mainly contain flavonoids, alkaloids, essential oil and coumarins [2,15]. Many experiments indicated that *citrus* flavonoids exerted multiple beneficial effects on cardiovascular and metabolic health through antioxidant, antidiabetic and anti-inflammatory activities, and modulating lipid metabolism and adipocyte differentiation, *etc*. [86,87]. Simultaneously, essential oil has extensive pharmacological activities to central nervous system, such as sedation, hypnosis, anti-anxiety and anti-depression, and also to digestive system including gastro- and hepato-protective [88]. Considering that the decoctions of these herbs were usually used for treating some diseases, the contributions of these ingredients for the pharmacological effects from the application of these herbs would be lower than the anticipation because of the weak hydrophilicity of many flavonoids, essential oil and coumarins in these herbs. Here, two water-soluble components stachydrine and choline present extensive biological activities, including beneficial effects on cardio-cerebrovascular and nervous systems, kidney, liver, blood, and obviously regulating uterus effect (pregnancy and non-pregnancy), *etc.* (Table 3). Taken together with the contents of stachydrine and synephrine in these herbs, and the balance from their pharmacokinetics after oral administration, it was inferred that this pair of equilibrists plays an unignorable role on the pharmacological effects of these herb decoctions.

Some reports indicated that aqueous extract or the decoction of these herbs can contract the *in vitro* and *in vivo* uteruses of both pregnant and non-pregnant rabbits, and while relax the *in vitro* uterus of pregnant rats or mice [89-91]. Ahangarpour, *et al*. confirmed that the aqueous extract of *C. aurantium* flowers can reduce spontaneous motility and decrease the uterus contractions of pregnant rats, relating with voltage dependent calcium channels and without involving β-adrenoceptors and opioid receptors [92]. From Table 3, many reports indicated that stachydrine can not only stimulate uterine contraction, and also inhibit uterine spasm, for both pregnancy and non-pregnancy rabbits [25,46,47,49]. Thereby, the discovery of stachydrine with good water-solubility and considerable content in these herbs can provide some reasonable interpretations for the heterogeneous effects of these herb decoctions on the uteruses, depending on the different contents of stachydrine and other related components in these herbs and the physiological states of uteruses. Another, choline has similar effects on the uterus with stachydrine [49], while its contents in these herbs are only about 1/5 to 1/25 of those of stachydrine (Table 2).

Traditionally, these herbs are used for the treatment of some cardiovascular disease, and have various beneficial effects for cardiovascular health, including antioxidant and anti-inflammatory, hypolipidemic, anti-thrombusis and anti-atherosclerosis ones, and cardio-cerebrovascular protection, and so on [3,93,94]. Recently, Mahmoud, *et al*., summarized that *Citrus* flavonoids confer cardiovascular protection via their antioxidant, antidiabetic, anti-inflammatory, anti-atherosclerosis and other biological activities [86,87], and it was reported that some citrus flavonoids such as a multi-methoxy flavonoid nobiletin have anti-hypertensive activity [6]. Simultaneously, Pontifex, *et al*. also pointed out that *Citrus* fruits should be encouraged within the diet for their potential neurological benefits [95]. However, they also recommended that further studies and clinical trials should be performed for evaluating the efficacy. Simultaneously, it is noteworthy that the poor bioavailability

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of many citrus flavonoids has been an anxiety for their systemic effects after oral administration, due to the combination of their degradation by intestinal bacterial enzymes, the poor hydrophobic nature of aglycone, the efflux of intestinal P-glycoprotein and the metabolism of cytochrome P450 [96-98]. Moreover, the concentrated distributions of some citrus flavonoids such as tangeretin and naringenin in kidney, lung and liver will also influence the actual effects on cerebralvascular system [99,100]. Thereby, these factors would reduce the actual effects of citrus flavonoids on cardio-cerebrovascular system although their beneficial effects were confident. Based on these, stachydrine with large bioavailability (above 90%) and good tissue distribution would play an important role in the cardio- and cerebral-protections, since it has extensive beneficial effects on cardio-cerebrovascular system (Table 3). Moreover, the rapid vascular relaxation, slowing heart rate and decreasing cardiac output, and increasing coronary and myocardial blood of stachydrine can also offset the possible adverse effects from synephrine and *N*-methyltyramine on the heart under non-drug purpose, and which was probably some reason that sometimes the blood-pressure presented inapparent or transient raise and even decline after oral administration of these herb decoctions for experimental animals [7,8]. Furthermore, Liu, *et al*. reported that choline also has anti-hypertensive and cardiovascular protective effects [101].

It was reported that the concentrated solutions (1.5 g dried herbs per milliliter) of these citrus herb decoctions have obvious *in vitro* antiplatelet aggregation activities on human platelet [102]. However, synephrine would increase the level of platelet [35]. Conversely, stachydrine can not only inhibit platelet aggregation, and also ameliorate platelet-mediated thrombo-inflammation [25,26,36]. Thereby, considering that stachydrine has good water solubility and bioavailability, and considerable contents in these herbs, it can be confirmed that stachydrine is an important component for the anti-platelet aggregation of these herb decoctions, although some flavonoids, such as hesperidin and naringin [103,104], were also reported to have inhibitory activity against platelet aggregation [86,87].

Another, choline is already used for the treatment of nonalcoholic fatty liver disease [44,105]. Thereby, it can be deduced that choline in the decoctions of these herbs especially *Qingpi* would play an important role in maintaining the function and health of liver [43,44]. Moreover, choline is also beneficial for cardiovascular and atherosclerosis diseases, and possibly neurological disorders [106,107]. Besides the protective effects of *citrus* flavonoids on cardiovascular system mentioned above, they also have beneficial effects on gastrointestinal health and function [13,14], and renal, hepatic and nervous protections [108-110]. Nevertheless, the two-way regulating effect of these herb decoctions on gastrointestinal smooth muscle should be resulted from the combinational effects of flavonoids, essential oil and synephrine [13,15,89]. Simultaneously, citrus flavonoids, coumarins, stachydrine and choline should play main roles on the neuroprotective effects of these citrus herbs [2,110], and while essential oil, γ-aminobutyric acid and synephrine should be responsible for various functions for central nervous system, such as sedation, hypnosis, anxiolytic, anticonvulsant and anti-depression [11]. Moreover, citrus flavonoids, essential oil and stachydrine in these herbs could bring out the antitussive and expectorant effects on respiratory system.

Taken above together, stachydrine should play an important role in the pharmacological functions of these citrus herbs, especially it can dual-directionally regulate the uteruses and has various beneficial effects on cardio-cerebrovascular system, blood, kidney and liver. Simultaneously, as a pair of bioactive equilibrists with synephrine, the cardio-cerebrovascular protection of stachydrine can counteract the possible cardiovascular risk brought out from synephrine, and which is very beneficial for the safe use of these citrus herbs. Moreover, together with the pharmacological activities of alkaloids (choline, synephrine, *N*-methyl tyramine and γ-aminobutyric acid), and *Citrus* flavonoids, essential oil and coumarins, these above can more scientifically and reasonably interpret the substance bases for various pharmacological effects of these citrus herb decoctions (shown on Figure 5). Conversely, the differences in the formation, content, water solubility, extractability, and pharmacokinetic characteristics of these components would lead to the efficient differences of these four citrus Chinese herbs.

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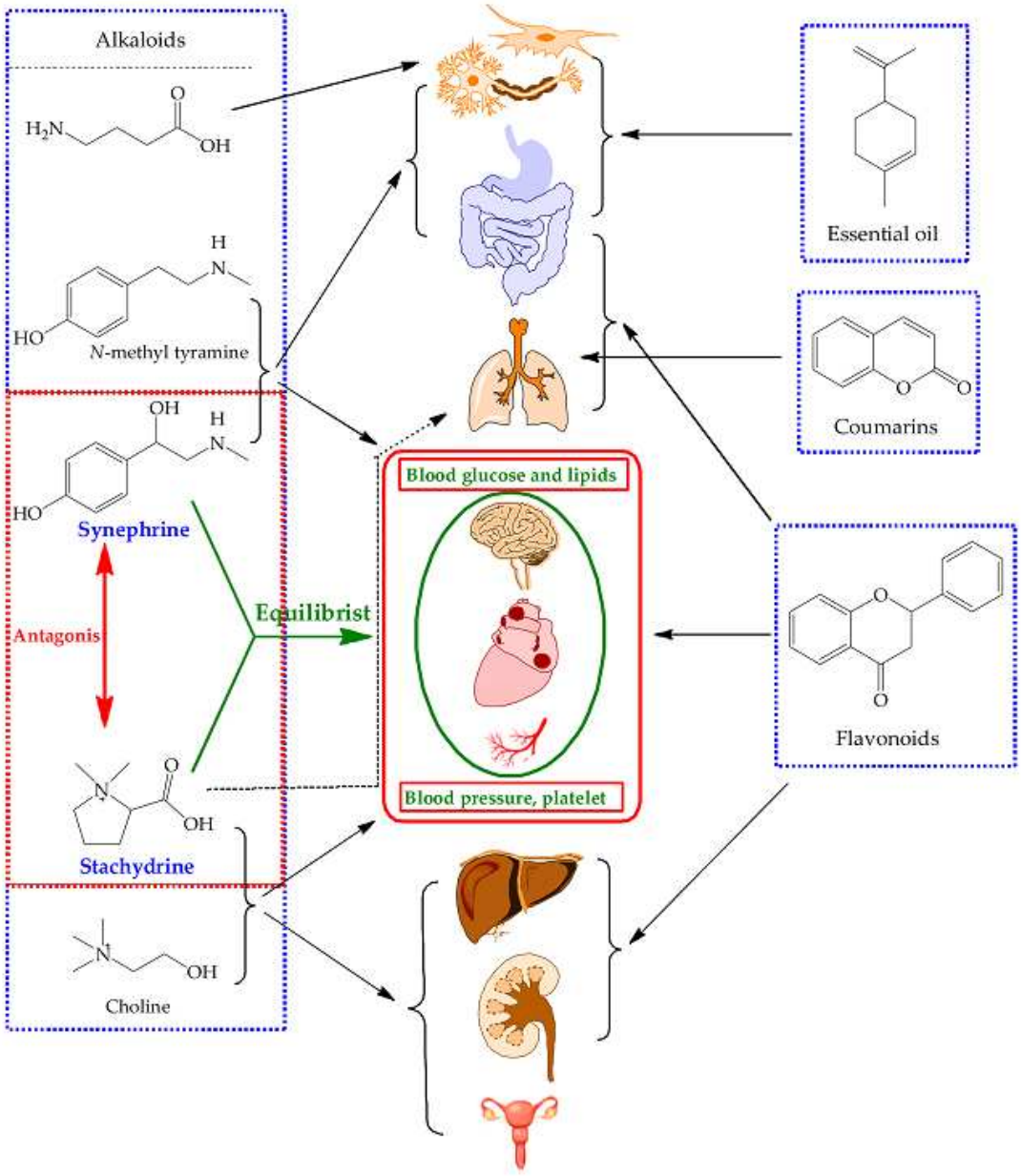

Figure 5. The ingredients for various pharmacological functions of the decoctions of four citrus herbs.

4. Materials and Methods

4.1. Materials, Chemicals and Reagents

Ten dried samples of *Zhishi*, *Qingpi* and *Chenpi* and nine samples of *Zhiqiao* from different places of production were purchased from Chengdu Huichu Technology Co., Ltd. (Chengdu, China). Moreover, eight leaf and fruit samples of *Citrus aurantium* L. and its cultivated varieties, *Citrus junos* Siebold ex Tanaka, and *Citrus reticulata* Blanco 'Zhangtouhong' were collected from different habitats and growth ages and were identified by senior agronomist Yao Nie, and some fresh leaves and fruits of *Xiangyuan* were collected at Xinyu in China. Chinese herbs *Foshou* as the dried fruits of *Citrus medica* L. *var. sarcodactylis* Swingle (Guangxi, China), *Xiangyuan* as the dried fruits of *Citrus wilsonii* Tanaka, *Huanglian* as the dried rhizome of *Coptis chinensis* Franch (Sichuan, China), *Chuanxiong* as the dried rhizome of *Ligusticum chuanxiong* Hort. (Sichuan, China), *Dafupi* as the dried peel of *Areca catechu* L. (Yunnan, China), *Banxia* as the processed products according to the legal process for the dried tuber of *Pinellia ternata* (Thunb.) Breit, *Juhua* as the dried capitulum of *Chrysanthemum*

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morifolium Ramat., *Mahuang* as Ephedrae Herba, *Duzhong* as the dried bark of *Eucommia ulmoides* Oliv., *Kushen* as the dried root of *Sophora flavescens* Ait., and *Gancao* as Glycyrrhizae Radix et Rhizoma were purchased from Yifeng Pharmacy (Xialuo Branch, Nanchang, China).

Reference standards choline chloride (No. C12799084) with a purity of 98% was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China), and stachydrine hydrochloride (No. PS012344) and synephrine (No. PS000966), with a purity of more than 98% for each, were purchased from Chengdu Push Bio-Technology Co., Ltd. (Chengdu, China).

TLC silica gel plates were purchased from Qingdao Ocean Chemical Co., Ltd. (Qingdao, China), and HPTLC Silica gel 60 F254 were purchased from Merck KGaA (Darmstadt, Germany). Bismuth subnitrate used for preparing the chromogenic reagent of TLC analyses were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Chromatographic grade methanol (Anhui Tedia High Purity Solvents Co., Ltd., China) and sodium dodecyl sulfonate (Baihe Chemical Plant, China) were used for the HPLC analyses of synephrine. The ultra-pure water was prepared by TST-UPB-10 ultra-pure water machine (Shijiazhuang TST Equipment Co., Ltd, China). Other chemicals (analytical purity) were purchased from China National Pharmaceutical Group Co., Ltd. (Beijing, China).

4.2. Detection of Choline Analogs

4.2.1. Controls and Chromogenic Reagents

Choline chloride was used as a positive control, and γ-aminobutyric acid and synephrine were taken as excluded controls. Two chromogenic reagents (Dragendorff's and Wagner) and an improved Dragendorff's one for alkaloids were selected for color reactions on the thin layer plate. The stock solutions were prepared by the equal mixture of solution I (0.85 g of bismuth nitrite dissolving in 10.0 mL of acetic acid and 40.0 mL of water) and solution II (8.00 g of potassium iodide dissolving in 20.0 mL of water), and 1.0 mL of the stock solution, 2.0 mL of acetic acid and 10.0 mL of water were mixed as Dragendorff's reagent before use. One gram of iodine and 10.0 g of potassium iodide were dissolved in 50 mL water, and then Wagner reagent was prepared by transferring this solution into a 100-mL volumetric flask, subsequently supplementing with 2.0 mL of acetic acid and required amount of water to make a constant volume. The improved chromogenic reagent was prepared by adding the mixture of bismuth nitrite 0.82 g, potassium iodide 11.06 g and 50% (*v*/*v*) phosphoric acid 90 mL into a 100-mL volumetric flask to make a constant volume using water.

4.2.2. Reference and Sample Solutions

A 25.0 mg of choline chloride, stachydrine hydrochloride and synephrine were respectively transferred into a volumetric flask, and then a concentration of 1.0 mg·mL^{-1} for three reference solutions was prepared by dissolving and supplementing with 80% (v/v) ethanol to a constant volume of 25 mL.

Fresh samples were cut into thin slices, and then dired under a 60°C of ambient temperature in a blast drying oven. All dried samples with appropriate amount were respectively crushed into coarse powder, and then passed through the sieve of 65 mesh to obtain correspoding powders of samples. A 2.000 g powder of each sample was placed into a 50-mL Erlenmeyer flask with a stopper which 25 mL of 80% (v/v) ethanol was added into. After sonicated twice for 10 min of each time in a DK-410T water bath sonicator with a frequency of 40 kHz, the mixture was filtered, and the residue washed twice with 80% (v/v) ethanol. The filtrate was concentrated under vacuum, and the residual suspension was transferred into a volumetric flask to prepare a 2 mL of sample solution with 80% (v/v) ethanol.

4.2.3. TLC analysis for Choline Analogs in Chinese Herbs

According to the general procedure of TLC analysis, the reference solutions of choline, γ-aminobutyric acid and synephrine were respectively sampled on a thin layer plate, and then developed with solvent systems I (ethyl acetate-95% ethanol-formic acid) and II (*n*-butanol-glacial

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acetic acid-water), respectively. After dried, the thin layer plate was visualized with Dragendorff's, Wagner and improved Dragendorff's reagents, respectively.

According to the optimized procedure, the TLC analyses for sample solutions from Chinese herbs *Zhishi*, *Zhiqiao*, *Qingpi* and *Chenpi* were performed for discovering probable choline analogs. After this, other herbs from *Citrus* genus including *Foushou* and *Xiangyuan*, and some samples of the leaves from the plants of *Citrus* genus were also detected for possible choline analogs. Moreover, to further verify the efficiency of the method detecting choline analogs, the TLC analyses for sample solutions from some other Chinese herbs, including *Huanglian*, *Juhua*, *Mahuang*, *Chuanxiong*, *Dafupi*, B*anxia*, *Duzhong*, *Kushen* and *Gancao* were also determined. Among these herbs, it was reported that herbs *Chuanxiong* and *Huanglian* contain choline or its analogs.

4.3. Isolation and Idengtification of Choline Analogs in Chinese Herbs Zhishi

According to the process of sample solution in section "4.2.2", a solution of the *Zhishi* mixed sample from 10 different producing areas was prepared. Using preparative TLC, probable choline analogs in Chinese herbs *Zhishi* were isolated. The solvent I (ethyl acetate-95% ethanol-formic acid, 10:4:5) was used as the developing agent, and probable choline analogs were located by the visualization of the slices of thin layer plates immersing the improved Dragendorff's reagent.

Compounds **1** and **2** were identified based on the spectral analyses for their NMR and MS, combining their physicochemical analyses. NMR data were recorded on a Bruker AV-600 or AV-400 MHz NMR spectrometer, and MeOH-d_4 and D_2O was respectively used as the solvent dissolving compounds **1** and **2**. Compound **3** was identified based on the spectral analyses for its HRMS and comparing the TLC and HPLC profiles of synephrine, compound **3**, and their mixture. All HRMS data were obtained with ESI ion source (positive ion mode) from a TripleTOF 5600^+ hybrid quadrupole time-of-flight mass spectrometer system (AB Sciex, USA).

4.4. Quantitative Analyses of Chlorine and Stachydrine with TLCS

4.4.1. Procedure of TLCS Analysis

Reference and sample solutions were prepared according to the procedure described in section "4.2.2.", and all of them were filtered by a 0.45-μm microporous membrane before use. A certain volume of sample solutions and reference solutions of choline or stachydrine was respectively sampled on a TLC plate placed on a YOKO-TD electric strip spotter (Whyoko New Technology Development Co., Ltd., China), and the plate was placed in a developing chamber. After saturated with the solvent of ethyl acetate-95% ethanol-formic acid (10:4:5, v/v/v) for 15 min, the spots were developed for a span length of 60 mm. Subsequently, the plate was taken from the chamber and dried, and then was immersed in the improved Dragendorff's reagent for 2 to 4 s. After taken from the stained jar for 20 min, TLCS analyses was performed with reflection absorption method in 50 min on a KH-3000 Plus TLC Scanner (Shanghai Keze Biochemical Technology Co., Ltd., China), and the measured/reference wavelengths for choline and stachydrine were 568/820 nm and 574/820 nm, respectively. The contents of choline or stachydrine in Chinese herbs from Citrus genus were calculated from the mean peak areas of three bands of choline or stachydrine from reference and sample solutions.

In the above procedure, the measured/reference wavelengths and the chromogenic stability were simultaneously made sure from the 10-min interval determination in 70 min for the absorption curves of choline and stachydrine bands on the TLC plate after visualized, and the sampling volume was 5.0 μL. Depending on the typical chromatographic profile of TLC analyses for sample solutions of citrus Chinese herbs, the resolution between choline (or stachydrine) and its nearest strip was calculated.

4.4.2. Methodology Validation

According to the general procedure of methodology validation, the limit of detection (LOD), limit of quantitation (LOQ), precision, repeatability, linearity and range were evaluated using the

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reference standard of choline or stachydrine. The reproducibility was assessed using the powder of Zhishi. The recovery was tested using the powder of Zhishi with a known content of choline or stachydrine, and the reference standard of choline or stachydrine.

In detailed, the LOD was defined as the lowest concentration of choline or stachydrine at which the signal-to-noise ratio was from 3 to 4, and the LOQ was defined as the lowest concentration of choline or stachydrine at which the signal-to-noise ratio was more than or equal to 10 with a precision below 5%. According to the procedure of TLCS analysis in subsection "4.4.1.", six scans to a strip of choline or stachydrine on a plate were performed for evaluating the repeatability, using a choline or stachydrine solution (1.0 mg·mL^{-1}), and the sampling volume was 10.0 μL. Simultaneously, intra- and inter-plate precision were assessed by detecting of choline or stachydrine solutions respectively on a thin layer plate with six replicates and on six thin layer plates. The linear correlation was established using seven sampling volumes (three replicates for each) at a concentration of 1.0 mg·mL^{-1} for choline or stachydrine solution. The reproducibility was evaluated by the quantitative determination of choline or stachydrine in Zhishi powder (No. 20101001) with six replicates, and the sampling volume was 2.0 μL. The recovery was tested from 9 samples prepared by adding an amount of choline or stachydrine into the powders of Zhishi (No. 20101001) with a known content of choline or stachydrine, including three different adding amounts for choline or stachydrine with three replicates for each.

4.4.3. Quantitative Analyses for Samples

According to the validated procedure of TLC analyses, the quantitative analyses of choline or stachydrine, in thirty-nine purchased samples including ten of Zhishi, ten of Qingpi, ten of Chenpi, and nine of Zhiqiao ones, were performed with the external standard method in triple for each sample. Three spots were sampled on the plate for each sample or standard solution. The sampling volume of each spot for the sample solutions of Zhishi was 2.0 μL, and while that for the sample solutions of Zhiqiao, Qingpi or Chenpi was 3.0 μL. Simultaneously, the sampling volumes were 1.0 and 3.0 μL for the reference solutions of choline and stachydrine, respectively. After visualized, each lane on the plates was scanned for obtaining the peak areas of choline or stachydrine in samples and corresponding standards, and then the contents of choline and stachydrine in various samples were respectively calculatd.

4.5. Quantitative Analyses of Synephrine with HPLC

4.5.1. Procedure of HPLC Analysis

A 5.0 mg·mL^{-1} of stock solution for synephrine was prepared by dissolving 10.0 mg of synephrine into 80% (*v*/*v*) ethanol, and transferred into a 2.0-mL volumetric flask which was further supplemented with 80% (*v*/*v*) ethanol to a constant volume. From this stock, a concentration of 0.5 mg·mL^{-1} for standard solution was prepared by 10 times dilution.

The concentrations of synephrine in sample solutions were determined with the external standard method, referring to the procedure of Chinese Pharmacopoeia [1]. Briefly, the quantitative analyses of synephrine were performed using a Waters e2695 separation system consisting of a model 2998 ultraviolet detector (Waters, MA, USA), and the detection wavelength was set at 275 nm. A SinoChrom ODS2 (4.6 mm × 250 mm, 5.0 μm) (Elite, Dalian, China) was used as the chromatographic column, and which the temperature was kept at 30°C. Methanol and phosphate buffer with the ratio of 67:33 (*v*/*v*) was used as the mobile phase, and the flow rate was set at 1.0 mL/min. The phosphate buffer was prepared by dissolving 0.60 g of KH_2PO_4, 1.00 g of sodium dodecyl sulfonate and 1.0 mL of glacial acetic acid into 700 mL of water, transferred into a 1000-mL volumetric flask, and subsequently supplementing with a required amount of water to make a constant volume. Moreover, the injection volume for all sample and standard solutions was 10.0 μL. Depending on the typical chromatographic profile of HPLC analyses for sample solutions of citrus Chinese herbs, the resolution between synephrine and its nearest chromatographic peak was automatically calculated by the HPLC system.

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4.5.2. Methodology Validation

Similar to the validation of TLC analysis, the LOD was defined as the lowest concentration of synephrine at which the signal-to-noise ratio was from 3 to 4, and the LOQ was defined as the lowest concentration of synephrine at which the signal-to-noise ratio was more than or equal to 10 with a precision below 5%. The linear correlation was established using seven concentrations (0.05, 0.10, 0.20, 0.40, 0.80, 1.60 and 3.20 mg/mL) of synephrine solution with three replicates for each. Six injections for standard solution with a concentration of 0.50 $mg \cdot mL^{-1}$ were performed to evaluate the repeatability. Using a powder sample of Zhishi (No. 2010001), the stability of a sample solution in 24 h was evaluated, and the reproducibility was also assessed by the quantitative determination of synephrine in the powder sample with six replicates. The recovery was tested from nine samples prepared by adding an amount of synephrine into the powders of Zhishi (No. 20101001), including three different amounts for synephrine with three replicates for each.

4.5.3. Quantitative Analyses for Samples

All sample solutions used for the quantitative analyses of chlorine and stachydrine in section "4.4." were simultaneously used for the quantitative analyses of synephrine according to the valid procedure.

4.6. Statistical Analysis for the Contents of Three Ingredients in These Four Citrus Chinese Herbs

All statistical Analysis were performed using EXCEL software, and bilateral t-test was used for the comparision of two groups. Paired t-tesst was selected when the number of data is same (both n = 10), while the two-sample equivariance was selected for t-test when that is different (n = 10 and 9). The P value less than to 0.05 showed the data difference of two groups is significant, and those less than to 0.01 indicated the data difference of two groups is very significant.

4.7. Comprehensive Analyses for Pharmacological Effects of Four Citrus Chinese Herbs

The bioactivities of synephrine, chlorine and stachydrine were unsystematically searched from Google academic search engine, and two databases Medline, CNKI and RSC in recent 10 years, using keywords "pharmacological" or "activity" or "review", and "synephrine", or and "chlorine", or and "stachydrine". Furthermore, the relevant references in the obtained literature were also tracked. Based on the resulted literatures and the contents of synephrine, chlorine and stachydrine in these four citrus Chinese herbs, the pharmacological effects of these compounds were comprehensively analyzed, together with their contributions to the pharmacological effects of four citrus Chinese herbs. Simultaneously, some reasonable explanations were also presented for the difference among the pharmacological activities of these four Chinese herbs.

5. Conclusions

To further clarify the reasons of the confusing functions of four citrus Chinese herbs, a simple and specific method for quickly discovering possible choline analogs was established based on the extended structure-activity relationships of cholinergic and anti-cholinergic agents, and stachydrine and choline were discovered from these citrus herb decoctions. Then, a TLCS method was first established for the quantitatively analyses of stachydrine and choline, and the contents of both two ingredients and synephrine in 39 samples were determined. The results showed that stachydrine and synephrine have commensurate contents in these herbs, and the contents of both two ingredients and choline in these herbs present similar changing trends along with the delay of harvest time. However, the contents of synephrine decrease most rapidly, while those of stachydrine decrease most slowly. Based on these above, the pharmacological activities and pharmacokinetics reported of stachydrine and synephrine were compared, and the results indicated that stachydrine and synephrine can be considered as a pair of bioactive equilibrists in these citrus herbs, especially for their effects on cardio-cerebrovascular system. Simultaneously, it confirmed that stachydrine should play an important role in the pharmacological functions, especially in dual-directionally regulating the uterus and various

beneficial effects on cardio-cerebrovascular system, kidney and liver, of these citrus herbs. Finally, taken these above and the pharmacological activities of other identified ingredients in these herbs together, some more scientifical and reasonable interpretations were presented for various pharmacological functions of these citrus herb decoctions, and which indicated that the differences of these components in the formation, contents, water solubility, extractability and pharmacokinetic characteristics would lead to the differences in the pharmacological functions of these citrus herbs.

Supplementary Materials: The supporting information includes Figure S1: Color development reactions of choline (**1**), synephrine (**2**) and γ-aminobutyric acid (**3**) on thin layer plates; Figure S2: The linearity correlations between the amounts (x) and peak areas (y), of stachydrine (**a**), choline (**b**) and synephrine (**c**); Figure S3. Detection of choline analogs in samples originated from *Citrus* genus plants on thin layer plates; Figure S4. Detection of choline analogs in samples originated from other plants on thin layer plates; Table S1: The recovery of stachydrine (n = 9); Table S2: The recovery of choline (n = 9); Table S3: The recovery of synephrine (n = 9).

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