Relevant effects of Taohong Siwu decoction on isolated rat aortic ring dependent on endothelium nitric oxide-cGMP pathway

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Abstract: Using rat thoracic aortic rings to test the relaxing effects of the 95% ethanol extract and aqueous extract of Taohong Siwu decoction (THSW) on endothelium intact or endothelium removed aortic rings. Results showed that the 95% ethanol extract (0.1, 1, 10, 100, 1000 $mg \cdot L^{-1}$) and aqueous extract (0.1, 1, 10, 100, 1000 $mg \cdot L^{-1}$) of THSW were able to relax the intact endothelium aortic rings pre-contracted by 10^{-6} $mol \cdot L^{-1}$ PE. 10^{-4} $mol \cdot L^{-1}$ L-NAME and 10^{-5} $mol \cdot L^{-1}$ methylene blue both were able to inhibit the relaxation other than indomethacin. For the endothelium removed aortic rings, potassium channel blocker $3 \times 10^{-3} mol \cdot L^{-1}$ tetraethylammonium chloride and 10^{-5} $mol \cdot L^{-1}$ glibenclamide had no effect on the relaxation effects caused by the 95% ethanol extract and aqueous extract of THSW. It could be concluded that the 95% ethanol extract and aqueous extract of THSW relax blood vessel by endothelium-dependent way.

Keywords: Taohong Siwu, thoracic aorta, rat, aortic ring, relaxation.

INTRODUCTION

Drug with the function of relaxing blood vessels are wildly used to treat cardio-cerebral vascular diseases (Boden *et al.*, 2015; Flynn, Bradford, & Harvey, 2016). For the discovery of this kind of drug, finding new lead compounds from natural plants is an important approach. Lots of traditional Chinese medicine prescriptions have many potential roles in the treatment of cardiovascular diseases (Fang *et al.*, 2017; Li *et al.*, 2017; Wang *et al.*, 2016). So, researches focus on the extracts or ingredients of these traditional Chinese medicine prescriptions stand a good chance to find new drugs for cardiovascular treatment.

Taohong Siwu decoction (THSW) was a traditional Chinese medicinal prescription originally described in an ancient Chinese medical book "Yi Zong Jin Jian" written by Qian Wu in Qing Dynasty (Scheme 1). It consists of six kinds of Chinese herbs, including *Radix Rehmanniae*, *Rhizoma Chanxiong*, *Radix Angelicae Sinensis*, *Semen Persicae*, *Radix Paeoniae Alba* and *Flos Carthami* (Scheme 1). THSW is widely used in oriental countries to treat women's diseases especially for irregular menstruation and primary dysmenorrheal (Liu *et al.*, 2012). Nowadays, lots of researches showed that it can improve microcirculation, treat coronary heart disease, reduce the injury induced by middle cerebral artery occlusion (Li *et al.*, 2015; Wu et al., 2011), and induce human umbilical vein endothelial cells proliferation, VEGF secretion, nitric oxide production (Jin *et al.*, 2010; Guo, 2006; Jin *et al.*, 2006; Xia *et al.*, 2007; Yen *et al.*, 2014; Yin *et al*., 2013). However, there are no researches about its effects and related mechanisms on the relaxation to blood vessel.

In this study used rat thoracic aortic rings to study the vasodilation of 95% ethanol and aqueous extracts of THSW. The purpose was to find which component of THSW (water-soluble component or fat-soluble component) has better relaxing effect on blood vessels. These results will be valuable for the further study on the constituents of THSW to cardiovascular diseases.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats, male (220 to 250 g), were provided by the Qinglongshan Experimental Animal Breeding Farm (Nanjing, China). All animals were housed in the standard laboratory conditions. All animal experiments and experimental protocol were approved by the Animal Ethics Committee of China Pharmaceutical University.

Drugs

Radix Rehmanniae, *Rhizoma Chanxiong*, *Radix Angelicae Sinensis*, *Semen Persicae*, *Radix Paeoniae Alba* and *Flos Carthami* (table 1) were bought from Nanjing Haiyuan Chinese Herbal Medicine Co., Ltd and identified by Professor Mian Zhang (School of Traditional Chinese Pharmacy, China Pharmaceutical University). The aqueous extract of THSW was made by extracting THSW three times with distilled water using reflux extraction, then concentrated under reduced pressure (Each gram of

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the extract is equivalent to 1.95g of the original medicinal herbs). The 95% ethanol extract of THSW was prepared by extracting THSW three times with 95% ethanol by reflux extraction, then concentrated under reduced pressure (Each gram of the extract is equivalent to 5.14 g of the original medicinal herbs); acetylcholine (ACh), phenylephrine (PE), L-NAME, indomethacin (INDO), glibenclamide (Gly), tetraethylammonium chloride (TEA) and methylene blue (MB) (Sigma Chemical Co.) were used in the experiments.

Analysis the content of paeoniflorin, ferulic acid and hydroxysafflor yellow A by HPLC

Sample Preparation

The 95% ethanol extract and the aqueous extract of THSW were dissolved in methanol and filtered by 0.45μm filter, then both extracts were injected into the HPLC.

Standard solution

Paeoniflorin, ferulic acid and hydroxysafflor yellow A were dissolved in 50% methanol separately, the concentration were 0.128 mg/ml, 0.201 mg/ml and 0.134 mg/ml.

Analysis condition

Waters 2695 HPLC was used in this study. This equipment contained an Agilent ZORBAX SB- C18 (250 mm × 4.6mm, 5μm) column. For the analysis of Paeoniflorin and ferulic acid, the acetonitrile (A) and 1% phosphoric acid solution (B) (V/V, 15:85) were used as the mobile phase. For the analysis of hydroxysafflor yellow A, the acetonitrile (A) and 1% phosphoric acid solution (B) (V/V, 10:90) were used as the mobile phase. The flow rate, injection volumn and column temperature were 1.0 $mL \cdot min^{-1}$, 10 μl and 30°C. The detection wavelength was set to 230 nm for Paeoniflorin, 321 nm for ferulic acid and 403 nm for hydroxysafflor yellow A.

Aortic ring preparation

After anaesthetized with ether, rats were euthanized by cervical dislocation. The thoracic aorta was removed and put into the ice-cold Kreb-Henseleit solution (NaCl 118 $mmol \cdot L^{-1}$, KCl 4.7 $mmol \cdot L^{-1}$, $MgCl_2$ 1.2 $mmol \cdot L^{-1}$, $CaCl_2$ 2.5 $mmol \cdot L^{-1}$, $NaHCO_3$ 25 $mmol \cdot L^{-1}$, KH_2PO_4 1.2 $mmol \cdot L^{-1}$, glucose 11 $mmol \cdot L^{-1}$, pH =7.4). The connective tissue of the aorta was removed, then cut into 3 mm aortic rings. For aortic rings without endothelium, the wooden tooth pick was used to remove the endothelial layer. The aortic rings were put into 37°C thermostatic bath bubbling the mixture of 5% CO_2 and 95% O_2. One side of the aortic ring was fixed to the thermostatic bath and the other side was related to tonotransducer (Chengdu Techman Software Co., Ltd). Vascular tension was recorded by the BL-420 Biological Functional System (Chengdu Techman Software Co., Ltd.). The baseline load of the aortic ring was 2 g. These aortic rings were treated with KCl (60mM) for 15min, then washed with Kreb-Henseleit solution every 20min for 3 times. Then the aortic rings were treated with KCl (60mM) the second time for 15min and then washed every 20 min with Kreb-Henseleit solution for 3 times (Shen *et al.*, 2013).

Test of vascular vitality

PE (10^{-6} $mol \cdot L^{-1}$) was used to contract the aortic rings, when constriction reached equilibrium, relaxed the aortic rings with different concentration ACh (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} $mol \cdot L^{-1}$). Those aortic rings relaxed to more than 80% by 10^{-5} $mol \cdot L^{-1}$ ACh was considered as endothelium is intact. The aortic ring relaxed less than 1% by 10^{-5} $mol \cdot L^{-1}$ ACh was considered as endothelium is completely removed (Guven *et al.*, 2011).

The relaxant effects and mechanisms of THSW aqueous extract on aortic rings with intact endothelium

The aortic rings with intact endothelium were randomly divided to 4 groups: 1. Control, 2. L-NAME, 3. INDO and 4. MB. L-NAME group, INDO group and MB group were added 10^{-4} $mol \cdot L^{-1}$ L-NAME, $10^{-5} mol \cdot L^{-1}$ INDO and $10^{-5} mol \cdot L^{-1}$ MB separately, kept these reagents with aortic rings for 25 min. Contracted the aortic rings with PE (10^{-6} $mol \cdot L^{-1}$), and then added THSW aqueous extract (0.1, 1, 10, 100, 1000$mg \cdot L^{-1}$), recorded vasorelaxation and vasoconstriction tension.

The relaxant effects and mechanisms of THSW 95% ethanol extract on aortic rings with intact endothelium

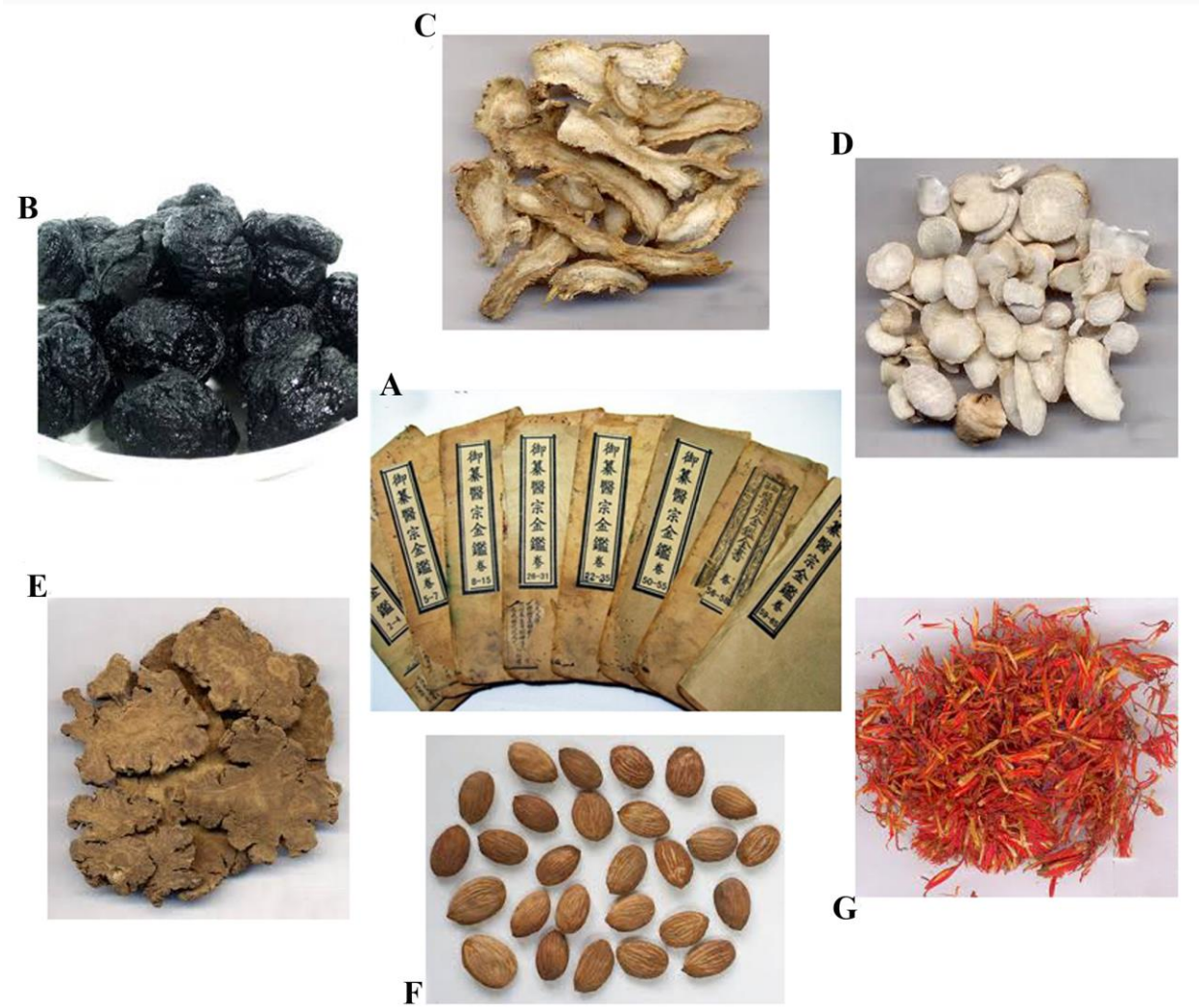
The aortic rings with intact endothelium were randomly divided to 4 groups: 1. Control, 2. L-NAME, 3. INDO and 4. MB. L-NAME group, INDO group and MB group were added L-NAME, INDO and MB separately, kept these reagents with aortic rings for 25 min. Contracted the aortic ring with 10^{-6} $mol \cdot L^{-1}$ PE, and then added THSW 95% ethanol extract (0.1, 1, 10, 100, 1000 $mg \cdot L^{-1}$), recorded vasorelaxation and vasoconstriction tension.

The relaxant effects and mechanisms of THSW aqueous extract on aortic ring without endothelium

The aortic rings without endothelium were randomly divided to 3 groups: 1. control, 2. Gly, 3. TEA. Gly group and TEA group were added 10^{-5} $mol \cdot L^{-1}$ Gly or $3 \times 10^{-3} mol \cdot L^{-1}$ TEA, kept these reagents with aortic rings for 25 min, Contracted the aortic ring with 1 $\mu mol \cdot L^{-1}$ PE, and then added THSW aqueous extract (0.1, 1, 10, 100, 1000 $mg \cdot L^{-1}$), recorded vasorelaxation and vasoconstriction tension

The relaxant effects and mechanisms of THSW 95% ethanol extract on aortic rings without endothelium

The aortic rings without endothelium were randomly divided to 3 groups: 1. control, 2. Gly, 3. TEA. Gly group and TEA group were added Gly or TEA, kept these reagents with aortic rings for 25 min, Contracted the aortic rings with 1$\mu mol \cdot L^{-1}$ PE and then added THSW



Scheme 1: "Yi Zong Jin Jian" (A); roots of *Rehmannia glutinosa* Libosch (B); *Radix Paeoniae Alba* (C); roots of *Paeonia lactiflora* Pall (D); *Rhizoma Chanxiong* (E)*; Semen Persicae*, seeds of *Prunus persica* (L.) (F); flowers of *Carthamus tinctorius* L. (G)

95% ethanol extract (0.1, 1, 10, 100, 1000mg·L^{-1}), recorded vasorelaxation and vasoconstriction tension.

STATISTICAL ANALYSIS

Results are presented as the mean ± SD. Student's t-test was used to determine the statistical significance of difference between the two groups. $p<0.05$ was considered statistically significant.

RESULTS

Determination of content of Paeoniflorin, ferulic acid and hydroxysafflor yellow A in the extracts

According to the results of HPLC (figs. 1, 2 and 3), the content of paeoniflorin, ferulic acid and hydroxysafflor yellow A in THSW aqueous extract were 8.2mg/g, 0.25mg/g, 2.55mg/g, respectively. The content of paeoniflorin, ferulic acid and hydroxysafflor yellow A in THSW 95% ethanol extract were 4.0mg/g, 0.76mg/g, 3.85 mg/g, respectively (table 2).

The relaxant effects of THSW aqueous extract on aortic rings with intact endothelium

THSW aqueous extract could diastolic the endothelium intact rings. The maximum relaxation was 66.95% (fig. 4). Pre-treatment with NG-nitro-l-arginine methyl ester (L-NAME) decreased this kind of relaxation. L-NAME treatment shifted E_{max} from 66.95% to 44.51%. The sGC inhibitor methylene blue (MB), shifted E_{max} from 66.95% to 44.36%. The indomethacin (INDO) treatment did not alter any relaxation by THSW aqueous extract (fig. 4E).

The relaxant effects of THSW 95% ethanol extract on aortic rings with intact endothelium

THSW 95% ethanol extract exerted vasodilatory effect on aortic rings with intact endothelium. The maximum relaxation was 51.81% (fig. 5). This kind of relaxation

Table 1: Component of THSW

Common name	Botanical name	Weight (g)
Radix Rehmanniae	Root of *Rehmannia glutinosa* Libosch (Scrophulariaceae)	12
Radix Angelicae Siensis	Root of *Angelicae sinensis* (Oliv.) Diels (Umbelliferae)	9
Radix Paeoniae Alba	Root of *Paeonia lactiflora* Pall. (Ranunculaceae)	9
Rhizoma Chaxiong	Rhizome of *Ligusticum chuanxiong* Hort. (Umbelliferae)	6
Semen Persicae	Seed of *Prunus persica* (L.) (Rosaceae)	9
Flos Carthami	Flower of *Carthamus tinctorius* L. (Compositae)	6

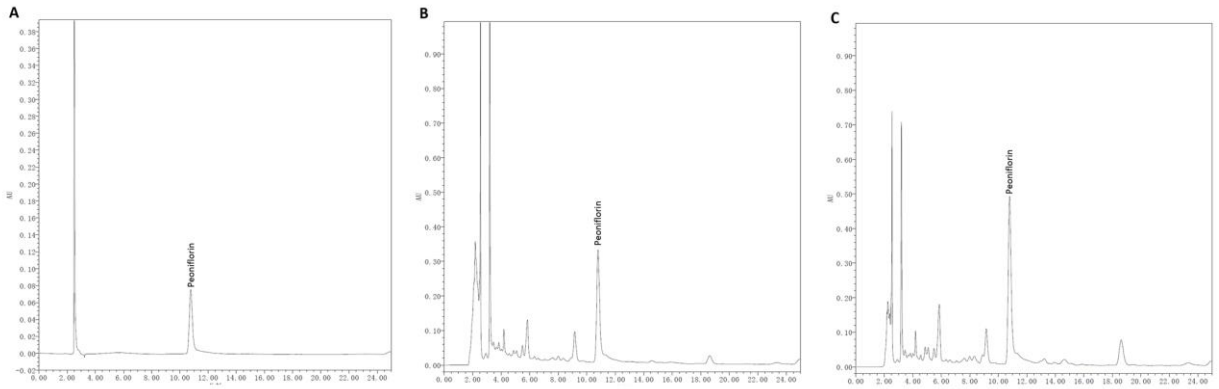


Fig. 1: HPLC of Paeoniflorin (A), aqueous extract of THSW (B) and 95% ethanol extract of THSW (C).

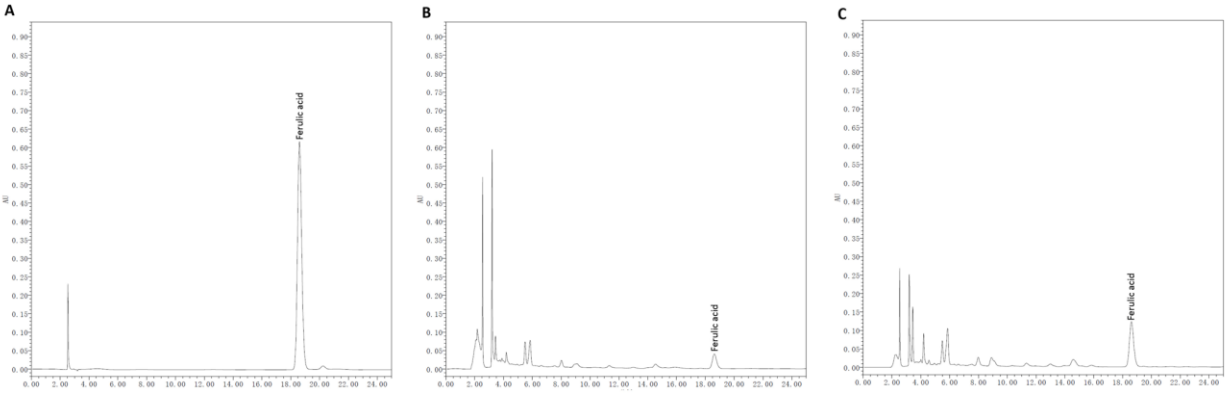


Fig. 2: HPLC of ferulic acid (A), aqueous extract of THSW (B) and 95% ethanol extract of THSW (C).

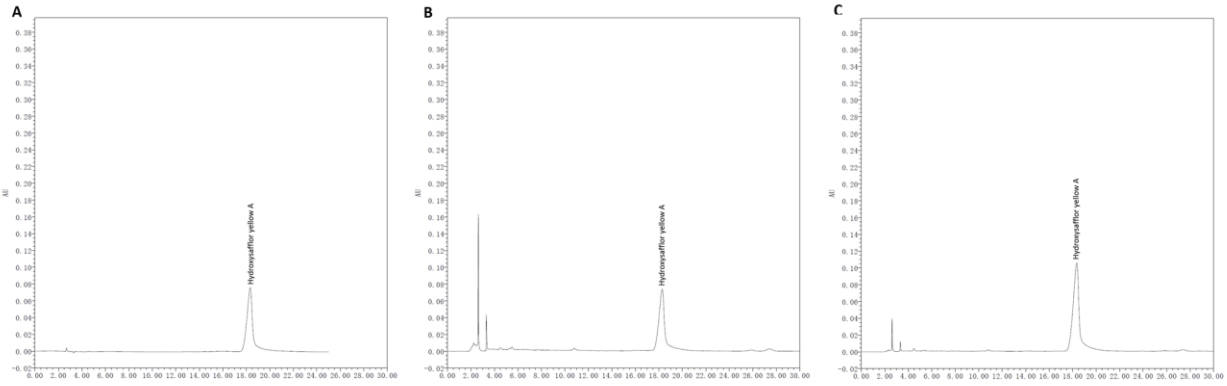
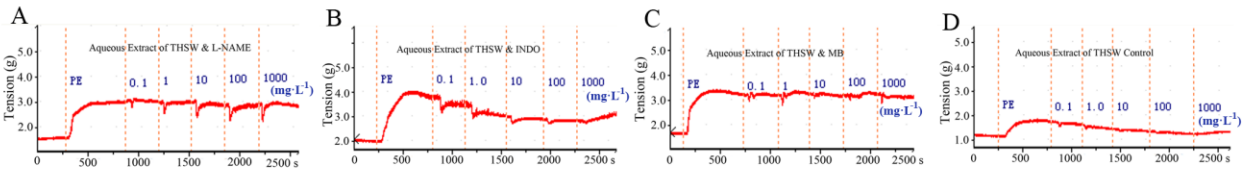


Fig. 3: HPLC of hydroxysafflor yellow A (A), aqueous extract of THSW (B) and 95% ethanol extract of THSW (C).

Table 2: The contents of paeoniflorin, ferulic acid and hydroxysafflor yellow A in the extracts (mg/g)

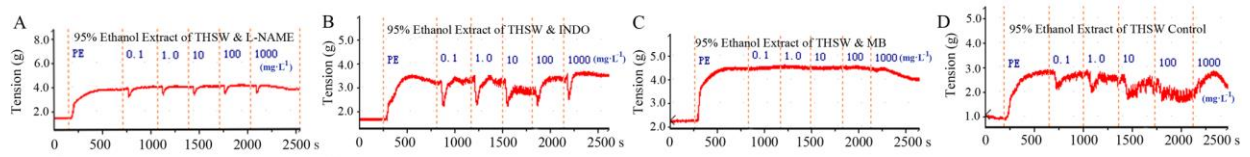
	Paeoniflorin	Ferulic acid	Hydroxysafflor yellow A
Aqueous extract of THSW	8.2	0.25	2.55
95% Ethanol extract of THSW	4.0	0.76	3.85



E

	0. 1 mg·L^{-1}	1 mg·L^{-1}	10 mg·L^{-1}	100 mg·L^{-1}	1000 mg·L^{-1}
Control	31. 74±12. 66	35. 34±15. 35	52. 03±10. 79	56. 51±18. 04	66. 95±15. 11
L-NAME	19. 01±6. 12*	28. 28±10. 17	38. 43±12. 23*	42. 33±14. 02	44. 51±9. 63**
INDO	43. 28±13. 35	48. 52±9. 44	54. 38±10. 48	58. 01±11. 66	67. 03±12. 66
MB	26. 34±18. 37	27. 63±13. 08	32. 39±15. 88*	31. 54±20. 10*	44. 36±20. 65*

Fig. 4: Relaxation curve of 10 $^{-4}$ mol·L^{-1} L-NAME (*n* = 10) (A), 10 $^{-5}$mol·L^{-1} INDO (*n* = 10) (B) and 10^{-5}mol·L^{-1} MB (n=10) (C) on aqueous extract of THSW induced relaxation and effect of 10 $^{-4}$ mol·L^{-1} L-NAME (*n* = 10), 10 $^{-5}$mol·L^{-1} INDO (*n* = 10) and 10^{-5}mol·L^{-1} MB (n=10) (E)on aqueous extract of THSW induced relaxation in the endothelium-intact aortic rings. Values are expressed as mean ± *SD*. **p*<0. 05, ** *p*<0. 01 vs Control.



E

	0. 1 mg·L^{-1}	1 mg·L^{-1}	10 mg·L^{-1}	100 mg·L^{-1}	1000 mg·L^{-1}
Control	44. 73±10. 57	52. 44±9. 39	55. 21±10. 83	52. 00±8. 93	51. 81±8. 78
L-NANE	21. 93±5. 73	21. 27±9. 09**	25. 53±8. 15**	22. 00±4. 87**	23. 74±3. 44**
INDO	26. 55±23. 28	36. 27±19. 00	38. 20±20. 81	40. 86±17. 84	41. 17±21. 16
MB	22. 17±10. 04*	24. 53±8. 31**	26. 51±6. 26**	32. 00±6. 00**	48. 32±14. 83

Fig. 5: Relaxation curve of 10 $^{-4}$ mol·L^{-1} L-NAME (*n* = 10) (A), 10 $^{-5}$mol·L^{-1} INDO (*n* = 10) (B) and 10 $^{-5}$mol·L^{-1} MB (n= 10) (C) on 95% ethanol extract of THSW induced relaxation and effect of 10 $^{-4}$ mol·L^{-1} L-NAME (*n* = 10), 10 $^{-5}$mol·L^{-1} INDO (*n* = 10) and 10 $^{-5}$mol·L^{-1} MB (n= 10) (E) on 95% ethanol extract of THSW induced relaxation in the endothelium-intact aortic rings. Values are expressed as mean ± *SD*. * *p*<0. 05,** *p*<0. 01 vs control.

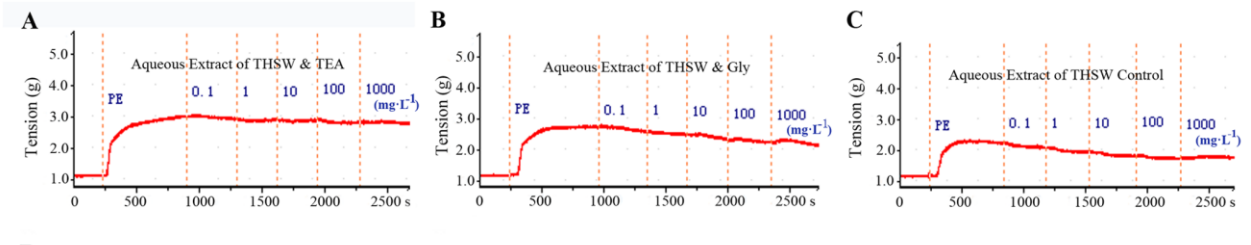
could be decreased by pre-treatment of L-NAME. L-NAME treatment shifted E_{max} from 51.81% to 23.74%. MB treatment shifted E_{max} from 52.00% to 32.00%. However, the relaxation induced by THSW 95% ethanol extract was not altered by pre-treatment with INDO (fig. 5E).

The relaxant effects of THSW aqueous extract on aortic rings without endothelium

Pre-incubated aortic rings preparations with Ca^{2+}-activated K^+ channels (K_{Ca}) blocker 10^{-4} M tetraethyl ammonium (TEA), K_{ATP} blocker 10^{-5} M glibenclamide (Gly) for 25 min respectively and found the relaxant effects of THSW aqueous extract could not be inhibited by those treatments (fig. 6).

The relaxant effects of THSW 95% ethanol extract on aortic rings without endothelium

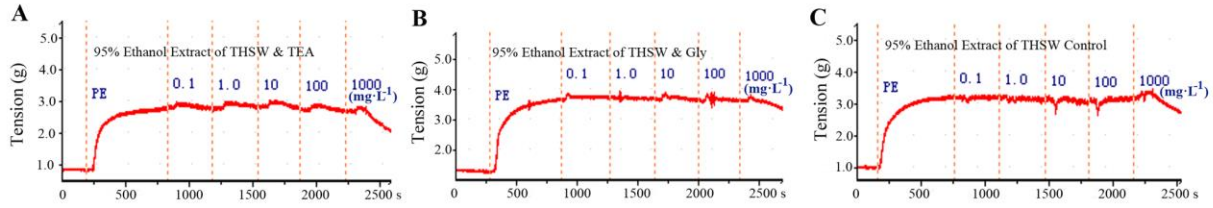
Pre-incubated aortic rings without endothelium with 10^{-4} M TEA or 10^{-5} M Gly for 25 min, that the relaxant effects of THSW 95% ethanol extract could not be inhibited by those treatments (fig. 7).



D

	0. 1 mg·L^{-1}	1 mg·L^{-1}	10 mg·L^{-1}	100 mg·L^{-1}	1000 mg·L^{-1}
Control	0. 45±1. 82	4. 62±5. 52	4. 56±6. 08	7. 22±5. 74	9. 45±3. 38
TEA	2. 04±3. 90	2. 98±8. 44	5. 05±8. 19	8. 49±8. 84	11. 08±7. 05
Glibenclamide	3. 02±4. 97	6. 47±8. 12	5. 80±3. 79	8. 86±3. 37	13. 66±1. 94

Fig. 6: Relaxation curve of 3×10 $^{-3}$ mol·L^{-1} TEA (n = 10) (A), 10^{-5} mol·L^{-1} Gly (n = 10) (B) on aqueous extract of THSW induced relaxation and effect of 3×10 $^{-3}$ mol·L^{-1} TEA (n = 10) and 10^{-5} mol·L^{-1} Gly (n= 10) (D) on aqueous extract of THSW induced relaxation in the endothelium- denuded aortic rings (g). Values are expressed as mean ± *SD*. * p<0. 05, ** p<0.01 vs Control.



D

	0. 1 mg·L^{-1}	1 mg·L^{-1}	10 mg·L^{-1}	100 mg·L^{-1}	1000 mg·L^{-1}
Control	2. 78±2. 90	3. 35±9. 38	5. 27±8. 14	7. 00±9. 13	26. 94±16. 91
TEA	0. 09±0. 77	4. 85±7. 65	5. 71±9. 28	8. 44±9. 81	30. 49±18. 67
Glibenclamide	−1. 89±2. 67	3. 39±6. 57	5. 54±6. 05	3. 26±6. 55	13. 63±7. 86

Fig. 7: Relaxation curve of 3×10 $^{-3}$ mol·L^{-1} TEA (n = 10) (A), 10 $^{-5}$mol·L^{-1} Gly (n = 10) on 95% ethanol extract of THSW induced relaxation and effect of 3×10 $^{-3}$ mol·L^{-1} TEA (n=10) and 10 $^{-5}$mol·L^{-1} Gly (n= 10) (D) on 95% ethanol extract of THSW induced relaxation in the endothelium- denuded aortic rings. Values are expressed as mean ± SD.

DISCUSSION

THSW was a traditional Chinese medicinal prescription, our previously study have proved that THSW was able to reduce the cerebral reperfusion injury of rat model (Chen *et al*., 2014). This study confirmed that THSW has a significant vasodilatation effect. The results of this study could contribute to the cerebral protection effects of THSW.

The results of present study proved that the 95% ethanol extract or aqueous extract of THSW induced a concentration-dependent vasodilator response to the rat aortic rings with an intact endothelium. The vasodilatation induced by the 95% ethanol and aqueous extract of THSW could be inhibited by removing endothelium. This proved that this kind of vasodilatation was endothelium-dependent. Then we used INDO, a prostanoid synthase cyclooxygenase inhibitor and L-NAME, the NO synthase inhibitor, to study the mechanisms. Results showed that INDO did not affect the vasodilatation induced by the 95% ethanol extract or aqueous extract of THSW. But L-NAME was able to inhibit the aqueous extract of THSW-induced vasodilatation. These results suggested that NO rather than prostacyclin was involved in the 95% ethanol extract or aqueous extract of THSW-induced endothelium-dependent vasodilation.

L-NAME is able to decrease the production of NO by inhibiting endothelial nitric oxide synthase (eNOS) (Chen *et al.*, 2009; Pedraza-Chaverrí *et al.*, 1998; Petros, Bennett, & Vallance, 1991); INDO is able to decrease the production of prostacyclin (PGI_2) by inhibiting cyclooxygenase (Curwen, Gimbrone, & Handin, 1980;

Weksler, Ley, & Jaffe, 1978), MB is able to decrease the production of cyclic guanosine monophosphate (cGMP) by inducing guanylatc cyclase (GC) (Duan *et al*., 2003; Li *et al.*, 2010; Rosenmund *et al*., 1994). Based on the results, it could be speculated that the aqueous extract and 95% ethanol extract of THSW induced the vasodilatation mainly by NO-guanosine monophosphate cyclase pathway.

Results also showed that the aqueous extract of THSW (0.1, 1, 10, 100 $mg \cdot L^{-1}$) or 95% ethanol extract of THSW (0.1, 1, 10, 100 $mg \cdot L^{-1}$) could not relax the aortic ring without endothelium. The aqueous extract of THSW (1000 $mg \cdot L^{-1}$) or the 95% ethanol extract of THSW (1000 $mg \cdot L^{-1}$) had the tendency to relax the rat aortic rings. But TEA, Gly both could not affect the vasodilatation caused by the aqueous extract of THSW (1000 $mg \cdot L^{-1}$) or the 95% ethanol extract of THSW (1000 $mg \cdot L^{-1}$). It has been proved that Gly can block ATP-sensitive K^+ (K_{ATP}) channels and TEA can antagonize broad range of K^+ channels (Pant, Ranjan, & Deshpande, 2011; Fernandes *et al.*, 2009). These results showed that K^+ channels, especially, K_{Ca} and K_{ATP} were not able to affect the pharmacological action of the aqueous extract or 95% ethanol extract of THSW.

Most of the Chinese herbal compound, include THSW, composed of several kinds of Chinese herbs with various ingredients. One of the advantages and characteristics of Chinese herbal compound is the synergistic effect of multiple components and multiple targets. The vasodilatation effect of THSW was also the synergistic effect of multiple components of THSW.

CONCLUSION

From this study, it could be concluded that vasodilatation induced by the aqueous extract and 95% ethanol extract of THSW based on their water-soluble and fat-soluble components, such as peoniflorin, ferulic acid, hydroxysafflor yellow A. Actually, the vasodilatation induced by peoniflorin, ferulic acid, hydroxysafflor yellow A have been confirmed by other researchers (Rhyu *et al.*, 2005; Tsai *et al*., 1999; Zhang *et al*., 2009). The vasodilatation effect of THSW is a synergistic effect of various effective components.

These results will be helpful for the components and mechanisms research About THSW extract for its vasodilatation effects.

ACKNOWLEDGEMENTS

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