

Original Article

Elucidation of Danzhixiaoyao Wan and Its Constituent Herbs on Antioxidant Activity and Inhibition of Nitric Oxide Production

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Danzhixiaoyao Wan (DW) is a common 10 herbs formulation in China for regulating several clinical conditions affecting women. This research tried to explain one of DW's functions, purging heat, using *in vitro* pharmacological analyses. The whole formulation and each single herb of DW were compared based on antioxidant activity with the oxygen radical absorbance capacity (ORAC) assay, and for their inhibitory effect (IE) on nitric oxide (NO) production by lipopolysaccharide (LPS)-activated RAW 264.7 macrophages with the Griess assay. The results showed that DW as a whole formulation had both antioxidant activity and an IE on NO production, while the individual herb component of DW varied in their ORAC values and inhibition of NO production. The ORAC value of the whole DW was 450 $\mu\text{mol TE g}^{-1}$. The order of antioxidant (ORAC) activity of the single herbs was: *Mentha haplocalyx* (1352 $\mu\text{mol TE g}^{-1}$) > *Glycyrrhiza uralensis* (1184 $\mu\text{mol TE g}^{-1}$) > *Gardenia jasminoides* (1129 $\mu\text{mol TE g}^{-1}$) > *Paeonia suffruticosa* (465 $\mu\text{mol TE g}^{-1}$), with the contributions being additive rather than synergistic. The production of nitrite by stimulated RAW 264.7 murine macrophages (unstimulated: $0.5 \pm 0.1 \mu\text{M}$ versus LPS: $38.9 \pm 2.3 \mu\text{M}$) was significantly inhibited ($P < 0.05$) by *M. haplocalyx*, *G. jasminoides*, *Bupleurum chinense* and *Paeonia lactiflora*. DW as a whole had an IE on NO production, but this was not significant. The single herb *M. haplocalyx* had the highest ORAC value and the highest IE on NO production, followed by *G. jasminoides*. Both of these herbs have the 'purging heat' property in the theory of traditional Chinese medicine and this property of the samples may be correlated with the antioxidant activity and IE on NO production.

Keywords: antioxidant activity – Danzhixiaoyao Wan – inhibitory effect on nitric oxide production – individual herbs – purging heat property

Introduction

Traditional Chinese medicine (TCM) belongs to Complementary and Alternative Medicine (CAM) in Western countries. TCM is based on a long tradition of herbal medicine, using single herbs, such as ginseng, or more often, multicomponent herbal formulations, such as *Shi quan da bu tang*. Such herbs and formulations are now being subjected to clinical research and pharmacology (1–3). Thousands of herbal formulations (generally consisting of 4–12 individual herbs) have been

recorded in Chinese medical literature from the earliest known '*Shen Nong Ben Cao Jing*' to the more contemporary Chinese *Materia Medica* (4,5). TCM may have fewer and less severe side effects than single pure drugs making TCM especially attractive to the consumers. A study on the use of CAM among American women showed that more than half the sample has used a CAM treatment or remedy, and 40% have visited a CAM practitioner (6). The use of CAM has increased in the United States over the past 20 years, reaching 36% in 2002; herbal medicine use accounts for ~22% of all CAM use (7). Chinese herbal medicines contain herbs, either singly or in mixtures, which are used for their scent, flavor and/or therapeutic properties.

Danzhixiaoyao Wan (DW) is a popular prescription to regulate women's disorders. It is from the famous

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prescription—Xiaoyao San (Ease Powder). The source of Xiaoyao San is *Taiping Huimin Hejiju Fang* (Benevolent Prescriptions from Taiping Pharmaceutical Bureau). The ingredients of Xiaoyao San are as follows: *Angelica sinensis*, *Paeonia lactiflora*, *Bupleurum chinense*, *Atractylodes macrocephala*, *Poria cocos*, *Glycyrrhiza uralensis*, *Mentha haplocalyx* and *Zingiber officinale*. Attributes of the constituents of Xiaoyao San in TCM are as follows: *B. chinense* is used to disperse stagnated liver-qi; *P. lactiflora* and *Angelica sinensis* are used to nourish blood for retaining yin and nourish the liver to relieve spasm; *P. cocos*, *A. macrocephala* and *G. uralensis* can supplement qi, strengthen the middle-energizer and reinforce the spleen to prevent the liver from subjugating it; *M. haplocalyx* assists to disperse the stagnated liver-qi, and *Z. officinale* helps to warm and regulate the middle-energizer. All the herbs used together disperse the stagnated liver-qi and enrich liver blood and restore the normal function of the spleen. In case of heat converted from stagnated liver-qi manifesting as a dry and bitter mouth, *Paeonia suffruticosa* and *Gardenia jasminoides* are also included in DW. The actions of DW are purging heat and soothing the liver, nourishing blood and invigorating the spleen (8).

In Chinese medicine, heat pertains to yang and relates to inflammation (9). When heat invades the body, it usually scorches the liver meridian, leading to the occurrence of liver-wind. In TCM theory, imbalance between yin and yang is a summation of all kinds of basic pathogenesis. DW can purge heat and reduce excessive yang, so it has yin characteristics. Studies have shown that the effective components of the yin-tonic herbs are mainly flavonoids with strong antioxidant activity (10–12). In addition, some of them are known to have anti-inflammatory properties (13). From recent research, antioxidants have a broad medicinal appeal because they have been linked to disease prevention and health maintenance characteristics for a number of conditions. Some Chinese formulations have been researched for their antioxidant activity. *Gui zhi fu ling wan* (a traditional Chinese formulation) improved microcirculation in patients with multiple old lacunar infarction prevented the progression of atherosclerosis and preserved endothelium-dependent vasorelaxation in cholesterol-fed rabbits by its antioxidant activity (14).

There have many reports of different test methods for measuring antioxidant effect. Here, we used the most popular, easiest and least expensive of the testing methods available: the oxygen radical absorbance capacity (ORAC) method. ORAC, an *in vitro* assay, measures antioxidant scavenging activity against peroxy radical produced by AAPH at 37°C. Fluorescein (FL) was measured as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxy radical. A high ORAC value indicates that the tested sample possesses a high potency of antioxidant activity.

Nitric oxide (NO) is a diatomic free-radical produced from L-arginine by constitutive and inducible nitric oxide synthases (cNOS and iNOS) in numerous mammalian cells and tissues. iNOS is induced by either bacterial lipopolysaccharide (LPS)

or a number of cytokines in macrophages, hepatocytes and endothelial cells. iNOS and NO are involved in host defense and immunity and modulate the inflammatory response (15,16). NO and its product may be generated in excess during the host response against viral and bacterial infections and contribute to some pathogenesis by promoting oxidative stress (17,18).

Our present study focused on the antioxidant activity of the DW and its constituent herbs as well as the effects of extracts of DW and constituent herbs on NO production in LPS-activated RAW 264.7 macrophages. Modern analytical methods are being used to substantiate pharmacological claims with pharmacological evidence for herbal formulations and their individual components. In this paper, we generated antioxidant and anti-inflammatory data in an attempt to explain the meaning of DW's TCM action—purging heat from stagnated liver-qi.

Methods

Preparation of Sample

The samples in this study were bought from Shanxi Provincial People's Hospital. The herbs were supervised by Shanxi Provincial Medicinal Materials Supervision Bureau using vouchered specimens. The herbs were dried and ground into powder.

Dried herbs (3 g; dried under high vacuum to constant weight) were placed in a centrifuge tube (20 ml) and mixed with water (10 ml). The solution was vortexed, steeped at room temperature (2 h) then sonicated (15 min). Extracts were centrifuged (14 000 r.p.m., 10 min) and the supernatants dried under high vacuum and the residue weighed. The dried extracts were redissolved in water at suitable concentrations for the assays.

The manufacture of DW involves mixing the dried ground herbs with water, compressing into pills and removing the water. The formula is listed in Fig. 1. Before testing, DW was ground in a mortar and pestle and 3 g of the dried DW was extracted as above.

ORAC Assay

The ORAC assay was performed essentially as described by Ou *et al.* (19). Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) and fluorescein disodium (FL, 3',6'-dihydroxy-spiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) were obtained from Aldrich (Milwaukee, WI). AAPH [2'-Azobis(2-amidinopropane) dihydrochloride] was purchased from Wako Pure Chemical, USA. Phosphate buffer solution was obtained 'in house'. All ORAC analyses were performed on a Victor 2 plate reader (Wallac) at 37°C with an excitation wavelength of 485 nm and emission wavelength of 535 nm. The area under the fluorescence decay curve for FL (AUC) was calculated by point-to-point integration. The 96-well polystyrene microplates were purchased from PerkinElmer Life and Analytical Sciences.

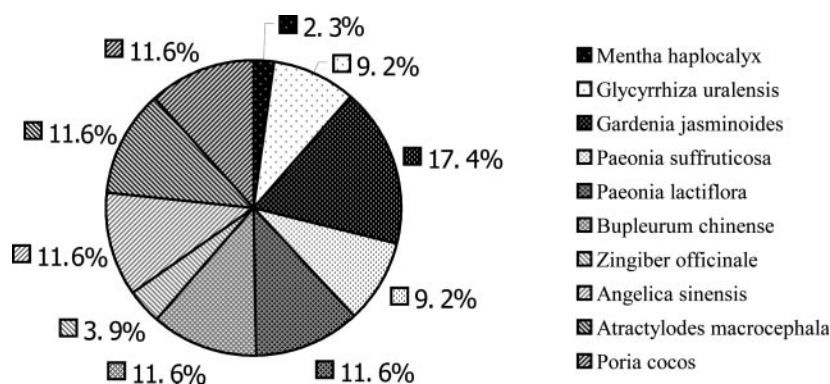


Figure 1. Herb list of the formulation [according to Chinese Pharmacopoeia (2000)] and the percentage of each herb.

The dried sample extracts were dissolved in water, and tested at concentrations of 12.5, 25, 50 and 100 mg extract per liter in triplicate. Briefly, 20 μl of sample or Trolox was incubated with 10 μl 75 nM FL and 170 μl 17 mM AAPH in a total volume of 200 μl . The decrease in fluorescence was followed at one minute intervals for 40 min at 37°C. The protective effect of an antioxidant was measured by comparing the AUC of the sample to that of a known antioxidant, Trolox, a water-soluble analog of vitamin E. The final ORAC values were expressed as micromole Trolox Equivalents per gram dried herb ($\mu\text{mol TE g}^{-1}$). The results were expressed as mean \pm SD.

NO Assay

iNOS activity was determined on RAW 264.7 cells from the American Type Culture Collection (Manassas, VA) cultured in color-free DMEM supplemented with 10% heat inactivated fetal bovine serum, D-glucose (3.5 mg ml⁻¹), Na pyruvate (100 mM), L-glutamine (2 mM) penicillin (100 U ml⁻¹), streptomycin (100 mg ml⁻¹) and amphotericin B (1 g ml⁻¹). Cells were plated at 2×10^6 per ml cells, 100 μl per well and maintained at 37°C in an atmosphere of 5% CO₂. Confluent cells in 96-well plates (Nunc) were pre-treated for 2 h with vehicle or samples prior to LPS (1 $\mu\text{g ml}^{-1}$) stimulation for 22 h. Nitrite, a stable endproduct of NO, was then measured using the Griess reaction (20). Supernatants of RAW 264.7 cells were mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid), followed by spectrophotometric measurement at 550 nm. Nitrite concentrations in the supernatants were determined by comparison with a sodium nitrite standard curve.

Results

ORAC Assessment of DW and Constituent Herbs

Table 1 reported the ORAC contributions of each constituent herb and the combination formula. The highest ORAC value was recorded for *M. haplocalyx* (1352 $\mu\text{mol TE g}^{-1}$), which is

Table 1. Antioxidant activity of DW and constituent herb

Chinese name	Latin name	ORAC ($\mu\text{mol TE g}^{-1}$)	ORAC% corr
Bo he	<i>Mentha haplocalyx</i>	1352 \pm 87	31
Gan cao	<i>Glycyrrhiza uralensis</i>	1184 \pm 68	109
Zhi zi	<i>Gardenia jasminoides</i>	1129 \pm 96	196
Dan pi	<i>Paeonia suffruticosa</i>	465 \pm 69	43
Bai shao	<i>Paeonia lactiflora</i>	224 \pm 12	26
Chai hu	<i>Bupleurum chinense</i>	175 \pm 14	20
Sheng jiang	<i>Zingiber officinale</i>	95 \pm 8	4
Dang gui	<i>Angelica sinensis</i>	88 \pm 10	10
Bai shu	<i>Atractylodes macrocephala</i>	61 \pm 5	7
Fu ling	<i>Poria cocos</i>	0	0
DW	Formulation	450 \pm 17	446

Antioxidant values for water extraction from each single herb expressed as mean \pm SD $\mu\text{mol TE g}^{-1}$. ORAC% corr referred to the ORAC value adjusted for the percent composition of each herb in DW as defined in Fig. 1.

higher than vitamin E (1162 $\mu\text{mol TE g}^{-1}$) but lower than vitamin C (2000 $\mu\text{mol TE g}^{-1}$) (21). Significant ORAC contributions are also made by *G. uralensis* (1184 $\mu\text{mol TE g}^{-1}$) and *G. jasminoides* (1129 $\mu\text{mol TE g}^{-1}$) whilst *P. cocos* did not show any antioxidant activity even at 400 mg l⁻¹. For the whole formulation, the ORAC value was 450 $\mu\text{mol TE g}^{-1}$ compared with the contribution of individual herbs at 460 $\mu\text{mol TE g}^{-1}$ corrected for weight composition of the formulation. From Fig. 2, we could see that *G. jasminoides* made the major ORAC contribution to DW, whereas *P. cocos*, *Z. officinale* and *A. macrocephala* contribute little to this aspect of the formulation. From this data the contributions appear to be additive with no evidence for synergy from the traditional combination formulation.

Inhibitory Activity on NO Production by DW and Constituent Herbs

As shown in Table 2, NO production increased dramatically from the basal level of $0.5 \pm 0.14 \mu\text{M}$ to $38.9 \pm 6.7 \mu\text{M}$ following stimulation with LPS. As shown in Table 2, with the

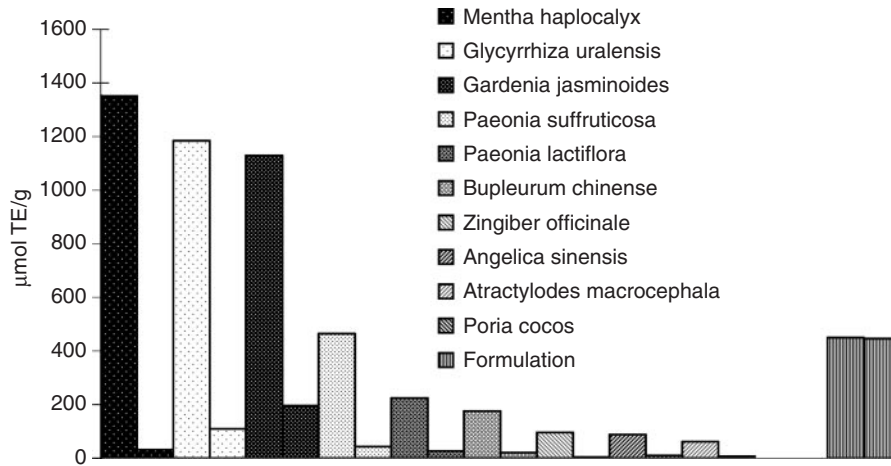


Figure 2. Antioxidant activity and ORAC% corr of constituent herb. For each herb, the first column showed antioxidant values ($\mu\text{mol TE g}^{-1}$), the second column ORAC % corr.

exception of *Z. officinale*, DW and the other nine herb extracts all reduced the LPS-stimulated NO production. Among them, *M. haplocalyx*, *G. jasminoides*, *B. chinense* and *P. lactiflora* had a significant effect when compared with LPS alone (Student's *t*-test, $P < 0.05$).

We compared the ability of 10 single herbs to reduce NO production. The inhibitory effect (IE) was expressed as the percentage decrease of NO production as

$$\text{IE (\%)} = \frac{100 - [\text{NO}]^a}{[\text{NO}]^b} \times 100,$$

where $[\text{NO}]^a$ represents the NO concentration in supernatants with sample added. $[\text{NO}]^b$ represents the NO concentration in supernatants from LPS-activated control macrophages (22). The IE is shown in Table 2.

Discussion

Chinese and Western medicines have both contributed greatly to health and disease treatment, but each has evolved its own fundamental rules. These rules make the two quite unique, in practice and in theory, especially in the drugs they design and use. Western medicine uses pure natural or synthetic compounds aimed at a single target, while TCM uses processed or crude multicomponent natural products, in various combinations and formulations aimed at multiple targets to treat a totality of different symptoms (23). DW is a combination of 10 herbs specific to the treatment of female disorders. In TCM, these disorders include hyperplasia of the lobule of the mammary gland, pre-menstrual tension, chronic pelvic inflammation and menopausal syndrome. Such disorders are caused by transformation of heat from liver depression, stagnation of liver-qi consuming blood, liver-wood depression subjugating spleen-earth and dysfunction of the spleen in transportation and transformation. DW is used to treat stagnation of liver-qi and deficiency of blood and

spleen, purging heat from stagnated liver-qi. This is marked by hypochondriac pain, headache, dizziness, dry mouth and throat, mental fatigue and poor appetite, or alternate attacks of chills and fever, or irregular menstruation, distension in the breast, reddish tongue, taut and thready pulse. DW is normally used as water-paste pills prepared by mixing the fine powdered herbs with excipients such as water to enhance a slow action and lasting effect (8). Laboratory water-paste pills were prepared as the sample for these antioxidant activity tests.

Some herbs that have high antioxidant activity are known to have anti-inflammatory properties (13). Also, inflammation is a typical symptom of excessive heat in TCM theory. That DW can reduce the excessive heat in TCM theory led us to investigate its antioxidant and anti-inflammatory activity.

DW had strong antioxidant activity, which would equate to 5400 $\mu\text{mol TE}$ per day taking into consideration its recommended dose of 12 g per day. It could suppress LPS-induced NO synthesis in RAW 264.7 macrophages by 28%. The combination of these pharmacological activities may explain the clinical actions of DW in purging heat in TCM.

In recent years there has been a trend that research would continue worldwide to identify and improve the efficacy of the active principals of herb both singly and in combination—from active ingredients, active fractions and active herbal formulations. There are 10 herbs in DW and the activity of the single herbs and their relationship with their TCM properties were also tested. According to TCM theory, three herbs in DW have the function of clearing heat and they are *P. suffruticosa*, *G. jasminoides* and *M. haplocalyx*. The results showed that *M. haplocalyx* and *G. jasminoides* had the highest antioxidant activity and significantly inhibited NO production.

It is interesting to note that *Z. officinale* had a different effect compared with other herbs whereby it seemed to stimulate the NO production at 500 mg l^{-1} ($235.4 \pm 33.0 \mu\text{M}$), but had no effect at 50 mg l^{-1} . As shown in Table 2 when the samples were at 500 mg l^{-1} , the significant IE were as follows:

Table 2. Effects of single herb and formulation on nitric oxide production of LPS-activated RAW 264.7 macrophages

Samples	Latin name	Concentration (mg l ⁻¹)	Nitrite (μM) ^a	IE (%)
Unstimulated			0.5 ± 0.1	
LPS			38.9 ± 2.3	
Bo he	<i>Mentha haplocalyx</i>	500	19.5 ± 0.6*	49.9
		50	25.1 ± 2.2*	35.5
Zhi zi	<i>Gardenia jasminoides</i>	500	20.9 ± 1.6*	46.3
		50	26.5 ± 4.2	31.9
Chai hu	<i>Bupleurum chinense</i>	500	36.6 ± 1.6	5.9
		50	24.6 ± 1.3*	36.7
Bai shao	<i>Paeonia lactiflora</i>	500	26.1 ± 1.3	32.9
		50	25.5 ± 1.2*	34.4
Dan pi	<i>Paeonia suffruticosa</i>	500	38.0 ± 5.1	2.3
		50	30.0 ± 2.4	22.9
Dang gui	<i>Angelica sinensis</i>	500	37.0 ± 2.2	4.9
		50	30.5 ± 0.9	21.6
Bai zhu	<i>Atractylodes macrocephala</i>	500	28.6 ± 5.4	26.5
		50	35.9 ± 6.0	7.7
Fu ling	<i>Poria cocos</i>	500	34.4 ± 1.6	11.6
		50	29.3 ± 2.9	24.7
Gan cao	<i>Glycyrrhiza uralensis</i>	500	31.0 ± 7.4	20.3
		50	28.8 ± 8.3	26.0
Sheng jiang ^b	<i>Zingiber officinale</i>	500	235.4 ± 33.0	
		50	35.7 ± 1.0	9.2
DW ^d	formulation	500	28.0 ± 3.7	28.0
		50	27.9 ± 5.6	28.3

^aData represent mean ± SD values from triplicate.

^b*Zingiber officinale* did not give IE at 500 mg l⁻¹.

*Denotes a significant effect of samples on LPS-induced nitrite concentration, compared with nitrite concentration in supernatants of cells stimulated with LPS alone ($P < 0.05$, Student's *t*-test).

M. haplocalyx (49.9%) > *G. jasminoides* (46.3%) > *B. chinense* (36.7%) *P. lactiflora* (34.4%).

Compound prescriptions, such as DW, are widely used based on a rational and appropriate compatibility of the herbs that contribute to total efficacy whilst reducing toxicity and side effects. However, the lack of quantitative data on herbal medications, makes it difficult to understand the potential for interactions (24). This research tried to understand the purging heat of DW with antioxidant and anti-inflammatory activity. DW showed its activity as did some of the constituent herbs. According to the principles of prescriptions, *G. jasminoides* and *P. suffruticosa* are the 'Monarch drugs' on purging heat, a Monarch drug being an essential ingredient in a prescription. It plays a leading curative role aiming at the cause or the main syndrome of a disease. In this prescription, *G. jasminoides* and *P. suffruticosa* played their role in purging heat. From Table 1, we can see that the ORAC% corr of *G. jasminoides* together with *P. suffruticosa* was 53% of whole DW's value. *G. jasminoides* is one of the heat clearing and fire purging

herbs (25). It showed the biggest contribution on ORAC% corr, and it also showed significant inhibition of NO production. The action of *P. suffruticosa* is to clear away heat, cool and circulate the blood to remove blood stasis. Whilst this herb showed relatively strong antioxidant activity (the ORAC% corr is the third one), there was inhibition of NO production, but it did not reach significance. There are some recent reports that crocin from *G. jasminoides* showed antioxidant properties, and a study of the reactions of crocin with linoleic acid and oxygen (26). Paeonol from *P. suffruticosa* was tested for antioxidative function *in vitro* (27). *M. haplocalyx* is one of three herbs in DW having the function of clearing heat, and it showed strong activity among these 10 herbs. This suggests some relationship between the TCM properties with antioxidant activity and NO inhibition in our research.

It is not surprising that purging heat in TCM theory is more complex than what we have observed in our study, but our research provides some evidence to understand the ancient theory. Understanding DW's TCM function in modern scientific terms is difficult. Other herbs that do not have a heat clearing function, such as *G. uralensis*, had the second highest value of ORAC% corr, as well as the second highest ORAC value. *B. chinense* and *P. lactiflora* had significant NO IEs. There are other actions of DW, including soothing liver, nourishing blood and invigorating the spleen that require further investigation.

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