

EXPERIMENTAL RESEARCH

Effects and Potential Mechanisms of Danzhi Xiaoyao Pill (丹栀逍遙丸) on Proliferation of MCF-7 Human Breast Cancer Cells *in vitro*

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ABSTRACT **Objective:** To investigate the effects of 50% ethyl alcohol (EtOH) extracts from Danzhi Xiaoyao Pill (丹栀逍遙丸, DXP) on the proliferation of MCF-7 human breast cancer cells and potential mechanisms. **Methods:** ATP-Lite assay was performed to test the proliferation of the MCF-7 breast cancer cell line; and antioxidant activity was measured by the oxygen radical absorbance capacity (ORAC). The effects of DXP on nitric oxide (NO) production were tested by lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages using the Griess reaction. **Results:** The 50% EtOH DXP extracts displayed a cytotoxic response on MCF-7 cells at 0.10, 0.25 and 0.50 mg/mL dose-dependently with the proliferation inhibited by more than 85%. The ORAC value of the DXP was 820 µmol Trolox equivalent/g, about 40% of the vitamin C value. DXP extracts had significant inhibitory effect on NO production at the concentration from 0.0625 mg/mL to 0.5 mg/mL ($P<0.05$, $P<0.01$). **Conclusion:** The extracts of DXP could significantly inhibit the proliferation of MCF-7 cells, with the effect possibly related to its antioxidant activity and the inhibition of NO production.

KEY WORDS Danzhi Xiaoyao Pill, breast cancer MCF-7 cells, antioxidant activity, oxygen radical absorbance capacity, nitric oxide

Traditional Chinese medicine (TCM) has special applications for the prevention and treatment of a variety of gynecological diseases, especially in treatment of breast cancer. A recent face-to-face structured interview of patients with advanced-stage breast cancer at some comprehensive oncology centers in the United States showed that Chinese herbal therapy is a treatment modality widely sought by breast cancer patients^(1, 2). Recent research indicated that many of the herbs often used in TCM have significant inhibitory effects on the growth of breast cancer cells⁽³⁾.

It was reported that women with mastoplasia have a 2-4 times higher risk of developing breast cancer as compared with those without mastoplasia, with 5% of those with mastoplasia developing cancer⁽⁴⁾. About 437 mastoplasia patients with risk factors were analyzed for types according to their syndrome differentiation by the TCM theory. The results showed that the percentage of patients with stagnation of the Gan (肝)-qi syndrome was 58.3% of the total, and this syndrome type was the one most often seen in mastoplasia patients⁽⁵⁾.

Danzhi Xiaoyao Pill (丹栀逍遙丸, DXP) could disperse the Gan (肝)-qi, nourish blood, and invigorate the Pi (脾) to promote transportation and transformation⁽⁶⁾. The following studies were conducted:

(1) the effects of 50% ethyl alcohol (EtOH) extracts from the DXP on the proliferation of MCF-7 human breast cancer cells; (2) the antioxidant activity of the extracts using oxygen radical absorbance capacity (ORAC); (3) the effect of extracts on nitric oxide (NO) production in lipopolysaccharide (LPS) activated RAW 264.7 murine macrophages.

METHODS

Preparation of Sample

The formula of DXP is shown in Table 1. DXP (3 g) was ground in a mortar and pestle, and the powder was suspended in 50% EtOH (10 mL), vortexed at room temperature for 2 h and extracted by ultrasound for 15 min. The supernatant was centrifuged at 5 000 g for 10 min, then dried under high vacuum and weighed. The extraction percentage calculated was $10.52 \pm 0.40\%$. The extracts were kept at 0-4 °C, and then redissolved in 50% EtOH at an appropriate concentration for the different assays.

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Table 1. The Formulae of DXP and the Percentage of Each Herb

Ingredient	Medicinal part	Chinese pinyin	Formula (%)
Paeonia suffruticosa	cortex	dan pi	9.2
Gardenia jasminoides	fruit	zhi zi	17.4
Angelica sinensis	root	dang gui	11.6
Paeonia lactiflora	root	bai shao	11.6
Bupleurum falcatum	root	chai hu	11.6
Atractylodes macrocephala	root	bai zhu	11.6
Poria cocos		fu ling	11.6
Glycyrrhiza uralensis	root	gan cao	9.2
Zingiber officinale	rhizoma	sheng jiang	3.9
Mentha haplocalyx	herb	bo he	2.3

Note: The formula is based on Chinese Pharmacopoeia (2000 ed)

Reagents

Giess reagent [0.1% N - (1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid] was prepared in-house. 2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), LPS and Giess reagent were purchased from Wako Pure Chemical, USA (Richmond, VA). The 6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid (Trolox, a water soluble homologue of vitamine E), pure vitamin C and 3', 6'-dihydroxy-spiro [isobenzofuran-1 [3H], 9'[9H]-xanthen]-3-one disodium (fluorescein; FL) were obtained from Aldrich (Castle Hill, NSW). The Victor 2 plate reader (Wallac) and the 96-well polystyrene microplates were purchased from PerkinElmer Life and Analytical Sciences.

Cell Source and Culture

MCF-7 cell lines were provided by the European Collection of Cell Cultures (Salisbury, UK). RAW 264.7 cell lines were purchased from the American Type Culture Collection (Manassas, VA). MCF-7 cell lines were routinely cultured in DMEM supplemented with heat inactivated fetal bovine serum (10%), L-glutamine (2 mmol/L), and penicillin (100 U/mL) media at 37 °C and 5% CO₂. RAW 264.7 cell lines were cultured in colorless DMEM supplemented with heat inactivated fetal bovine serum (10%), D-glucose (3.5 mg/mL), Na pyruvate (100 mmol/L), L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 mg/mL) and amphotericin B (1 g/mL) at 37 °C and 5% CO₂.

Effects of DXP on MCF-7

MCF-7 cell lines at 50%-70% confluence were used for plating for growth inhibition assays. For the

cytotoxicity assay, cells were steadily growing in a 96-well plate at 4 × 10⁴ cells/mL.

DXP sample (1 μL) or solvent control (50% EtOH, 1 μL) was added to cell suspension (99 μL) and cultured at 37°C in 5% CO₂ for 72 h. Viable cell count was determined using ATP-Lite test 72 h later. DXP extracts were tested at concentrations of 0.1, 0.25, and 0.5 mg/mL, and each concentration was assayed 9 times and the results expressed as mean ± standard deviation. Percentage of MCF-7 growth inhibition (%) = (1 – viable count in the DXP group/viable count in the control group) × 100%.

Determination of Antioxidant Activity

ORAC assay was performed as previously described⁽⁷⁾. Briefly, ORAC, an *in vitro* assay, measures antioxidant activity against peroxy radical produced by AAPH at 37 °C. The rate of loss of fluorescence of fluorescein (FL), the fluorescent probe, was measured every minute for 35 min, as an indication of the extent of damage from its reaction with the peroxy radical. The area under the curve (AUC) of fluorescence decay was calculated by point to point integration using Workout software (Wallac, Turku, Finland). AUC values for the samples was then quantified by comparison with the Trolox standard curve. The results were expressed as mean ± standard deviation. A high ORAC value indicates that the tested sample possesses a high antioxidant activity.

The Trolox standard was assayed at concentrations of 6.25, 12.5, 25, and 50 μ mol/L. The equation of the Trolox standard curve, prepared by graphing Trolox concentration versus AUC, was: Y (AUC) = 1.0259X (Trolox concentration) + 0.0960. Vitamin C, the positive control, was assayed at concentrations of 12.5, 25, 50, and 100 mg/L, in triplicate. The DXP samples were assayed with the experimental concentrations, methods, and calculation the same as those for vitamine C. The results were expressed as ORAC value per gram of DXP extract.

Determination of Effects of DXP on NO Content

Model preparation⁽⁸⁾: Inducible nitric oxide synthase (iNOS) activity was determined on RAW 264.7 cells (99 μL, plated at 2 × 10⁶ cells /mL) after stimulation with LPS (1 μg/mL). Nitrite, a stable end-product of NO metabolism, was then measured after 22 h using the Griess reaction⁽⁹⁾. Culture media of the RAW 264.7 cells was

mixed with an equal volume of Griess reagent, followed by the spectrophotometric measurement at 550 nm. Nitrite concentrations in the culture media were determined by comparison with a sodium nitrite standard curve.

For the solvent control, 50% EtOH (1 μ L) was added to the cell suspension (99 μ L) and distilled water (1 μ L) was added after 2 h. Sodium nitrite was tested after 22 h. The procedure for the LPS control was the same except that LPS (1 μ L) was added after 2 h instead of water. For the DXP extracts, sample (1 μ L) was added to the cell suspension (99 μ L) and LPS (1 μ L) was added after 2 h, before testing nitrite after 22 h. The samples were tested at the following concentrations: 0.0625, 0.125, 0.25 and 0.5 mg/mL. Each concentration was assayed 9 times and the results expressed as mean \pm standard deviation.

Statistical Analysis

All data were analyzed with SPSS software (SPSS Inc, Chicago, IL). Student's *t*-test was used for inter-group comparison.

RESULTS

In vitro Inhibitory Effect of DXP on MCF-7 Breast Cancer Cell Line Growth

DXP extracts markedly inhibited the growth of MCF-7 cell line in the range of 0.1 - 0.5 mg/mL (Table 2).

Table 2. Antiproliferative Activities in MCF-7 Breast Cancer Cells

Group	Concentration (mg/mL)	n	Percentage of growth inhibition (%)
Control		9	0
DXP	0.5	9	97 \pm 2*
	0.25	9	92 \pm 2*
	0.1	9	85 \pm 3*

Note: * $P<0.01$, compared with the control group

Antioxidant Effects of DXP

The ORAC value of DXP was $820 \pm 56 \mu\text{mol Trolox equivalent/g}$ dried sample (TE/g). This was 40% of the value for pure vitamin C ($1980 \pm 80 \mu\text{mol TE/g}$), indicating its comparably high level of antioxidant activity.

Inhibitory Effect of DXP on NO Production

NO production was increased significantly by LPS stimulation compared to the solvent control (50% EtOH, $P<0.01$). The extracts of DXP reduced the LPS-stimulated NO production significantly at all four concentrations as compared with LPS control ($P<0.05$ or $P<0.01$, Table 3).

Table 3. Inhibitory Effect of DXP on LPS-induced NO Production

Group	Concentration (mg/mL)	n	Nitrite ($\mu\text{mol/L}$)
50% EtOH control		9	$0.50 \pm 0.14^{**}$
LPS control		9	34.68 ± 1.06
DXP	0.5	9	$9.41 \pm 1.73^{**}$
	0.25	9	$10.52 \pm 1.26^{**}$
	0.125	9	$22.53 \pm 2.12^*$
	0.0625	9	$22.02 \pm 1.57^*$

Notes: * $P<0.05$, ** $P<0.01$, compared with the LPS group

DISCUSSION

Alternative medicine has gained increasing attention in Western countries, especially in cancer treatment. In the realm of botanical medicine, Chinese herbal therapy also enjoys favor from many cancer patients and doctors in this sector. But some Chinese medicines, though extensively applied in clinics, are cautiously used by Western medical colleagues due to lack of relevant pharmacological data. The absence of knowledge of the mechanisms of action of botanicals has been cited as an obstacle to the pharmacological development of botanical agents⁽³⁾. The authors hope to better understand DXP's clinical application through the preliminary exploration of its pharmacological effects.

To provide soluble extracts compatible with *in vitro* assays of DXP, we utilized 50% EtOH extracts as the solvent in the three assay systems. It is not appropriate to directly attribute these *in vitro* activities to clinical utility, but the result highlights the need to gain greater understanding of the pharmacological profile of natural products. Our present research used DXP extracts to test the antiproliferative effect of DXP on MCF-7, the breast cancer cell line. DXP showed cell toxicity on the MCF-7 cell line, with 97% growth inhibition by the EtOH extract at 0.5 mg/mL, and 85% growth inhibition at 0.1 mg/mL.

Multistage carcinogenesis is closely related to peroxidative state and excessive NO production. The utilization of antioxidants has been recognized in the treatment of many diseases⁽¹⁰⁾. Overproduction of free radicals may damage cells, resulting in a series of pathological processes, such as heart disease, cancer and aging. Excessive *in vivo* NO may also induce many pathological effects, such as inflammation, cell cycle arrest, and early cell apoptosis. Furthermore, carcinogenesis may result from mutational events

following NO-mediated DNA damage and hindrance to DNA repair. In a majority of human and experimental tumors, tumor-derived NO appears to stimulate a rapid growth of tumors; however, the opposite effect may appear under minor conditions⁽¹¹⁾.

NO, free radicals, superoxide (O^{2-}), and their reaction product peroxynitrite ($ONOO^-$) may be generated excessively during the host response against viral and bacterial infections, and contribute to some pathological processes by enhancing oxidative processes in the organism, leading in turn to oxidative stress, tissue injury and even cancer^(12,13). Conversely, a substance with definite anti-oxidative and anti-inflammatory properties has a potential anti-tumor activity⁽¹⁴⁾.

There are many reports of different test methods for measuring antioxidant activities of Chinese medicine. In this study we used the ORAC method, the most popular, easy and least expensive testing method described by Ou, et al⁽⁷⁾ which determines antioxidant values on foods, supplements and herbs. The results showed that DXP had a comparatively high ORAC value. Its ORAC value was 820 $\mu\text{mol TE/g}$, which was 40% of that of pure vitamin C.

We investigated the effects of DXP on NO production in LPS-stimulated mouse macrophages (RAW 264.7). The results showed that DXP EtOH extracts, in the concentration range of 0.0625-0.5 mg/mL, significantly reduced LPS-stimulated NO production in RAW 264.7 macrophages.

From our results, DXP displayed antiproliferative activities in MCF-7 breast cancer cells, suppressed LPS-induced NO production in RAW 264.7 macrophages and had moderate antioxidant activity. Recent research has shown that DXP can regulate the genital and endocrine system in rabbits *in vivo*⁽¹⁵⁾. There is a need for further investigations into the relationship between DXP's antioxidant, anticancer and NO modulating activities and its regulation of the endocrine system. This would give more insight into the mechanism of action of DXP.

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