

EXTRACTS OF *SALVIA MILTIORRHIZA* BUNGE ON THE CYTOKINES OF RAT
ENDOMETRIOSIS MODELSZan-Hua Zhou¹, Qing Weng², Jian-Hong Zhou^{3*}, Jue Zhou^{4*}¹School of Medicine, Lishui University, Lishui, 323000, Zhejiang, China²The Third People's Hospital of Yuhang District, Hangzhou, Zhejiang, 311115, China³Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, 310006, Zhejiang, China⁴College of Food Science and Biotechnology Engineering, Zhejiang Gongshang University, Hangzhou, Zhejiang, 310035, China.Email: juezhou2006@yahoo.com.cn**Abstract**

Endometriosis is a common mysterious and fascinating gynaecological condition with diverse clinical manifestations, highly variable and unpredictable clinical course with decreased quality of life. Clinically, *Salvia miltiorrhiza* Bunge (SMB, Chinese *Danshen*) has been applied to treat endometriosis and get satisfactory results. The present study was aimed to explore the effects of the extracts of SMB (ESMB) on the serum levels of cancer antigen 125 (CA-125) and the levels of interleukin (IL)-13, IL-18 and tumor necrosis factor-alpha (TNF-alpha) in the peritoneal fluids of rat endometriosis models. Three extraction methods for SMB were compared, which are the sample extracted with conventional method, the sample extracted with espresso coffee machine and the commercial condensed powder of natural products. We determined tanshinone IIA, salvianolic acid B and danshensu in the ESMB of different extraction methods. Forty female Sprague-Dawley (SD) rats were randomly divided into ESMB group, Danazol (positive control) group, model group and the sham-operation group (Sham group). After all the treatment ended, the serum levels of CA125 and the levels of IL-13, IL-18 and TNF-alpha in the peritoneal fluids of rat endometriosis models were measured using enzyme-linked immune-sorbent assay (ELISA) as directed by the manufacturer. The extraction efficiency of the ESMB samples extracted with coffee machine ranged from 600µm to 710µm was the highest. The serum levels of CA-125 and the levels of IL-18 and TNF-alpha in the peritoneal fluids of ESMB group, Danazol group and Sham group were significantly lower than those of the Model group ($P<0.05$). The serum levels of CA-125 and the levels of IL-18 and TNF-alpha in the peritoneal fluids of Danazol group and ESMB group were significantly higher than those of Sham group, respectively ($P<0.05$), and no marked difference existed between them ($P>0.05$). The levels of IL-13 in the peritoneal fluids of ESMB group, Danazol group and Sham group were significantly higher than those of the Model group ($P<0.05$). The levels of IL-13 in the peritoneal fluids of ESMB group and Danazol group were significantly lower than those of Sham group ($P<0.05$), and there was no marked difference between ESMB group and Sham group ($P>0.05$). ESMB shows promises in treating endometriosis by markedly decreasing the serum levels of CA-125 and the levels of IL-18 and TNF-alpha in the peritoneal fluids and significantly increasing the levels of IL-13 in the peritoneal fluids.

Keywords: Endometriosis, *Salvia miltiorrhiza* Bunge (SMB), Tanshinone IIA.**Introduction**

Endometriosis is a common mysterious and fascinating gynaecological condition with diverse clinical manifestations, highly variable and unpredictable clinical course with decreased quality of life (Okeke et al., 2011).

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<http://dx.doi.org/10.4314/ajtcam.v9i3.2>

Endometriosis affects approximately 10% of women of reproductive age (Giudice and Kao, 2004; Okeke et al., 2011). It most commonly afflict women in the late 20s and 30s and the classic symptom complex include dysmenorrhoea, dyspareunia, menorrhagia and infertility (Okeke et al., 2011).

Salvia miltiorrhiza Bunge (SMB, Chinese *Danshen*), is one of the most important Chinese medicinal plants, which is ranked as a “Supergrade” medicine in the first official book on Chinese herbal drugs, Shen Nong Materia Medica. SMB has been listed in the official Chinese Pharmacopoeia for treatment of menstrual disorder and blood circulation diseases and for prevention of inflammation (Kuang et al., 2005). Major compounds involved in its activity are quinoid diterpenes called tanshinones (tanshinones I, IIA, IIB, cryptotanshinone) and several phenolic acids (salvianolic, rosmarinic, caffeic, protocatechuic and danshensu acids)(Matkowski et al., 2008). Tanshinone IIA, salvianolic acid B, and sodium danshensu in SMB are usually used as bioactive markers in biological samples or herbal medicinal preparations (Zhou et al., 2005).

About 30% of the patients are asymptomatic and the incidence of infertility amongst women suffering from endometriosis ranges from 30%-40% (Okeke et al., 2011). The endometriosis-associated inflammatory response, tissue repair and neo-vascularization are dependent on the peritoneal fluids macrophages and their secretory products/cytokines (Fassbender et al., 2011; Huhtinen et al., 2011; Liu and Lang, 2011; Witz, 2002). These cytokines may play major roles in regulating cell proliferation, activation, motility, adhesion, chemotaxis and morphogenesis in the pathogenesis of endometriosis (Fassbender et al., 2011; Huhtinen et al., 2011; Liu and Lang, 2011; Witz, 2002). Increased peritoneal fluids concentration of cytokines that lead to the migration, proliferation, and activation of macrophages have been reported in patients with endometriosis (Sharpe-Timms et al., 1994; Weil et al., 1997). Although surgical and hormonal treatment are applied as the common interventions, the unpleasant side effects and high rates of relapse limited the range of these interventions (Abbott et al., 2004; Flower et al., 2009). Some medicinal plants were shown to be potential treatment for the disease (Flower et al., 2007; Wieser et al., 2007), and clinical trials have shown the eminent curative effects of some Chinese medicinal plants on the disease (Flower et al., 2009). Salvianolic acid B and Danshensu, 3-(3,4-dihydroxyphenyl) lactic acid are two natural phenolic acids of caffeic acid derivatives isolated from SMB. These two components have been reported to have potential protective effects from oxidative injury (Zhao et al., 2008). Tanshinone IIA, the major lipid-soluble pharmacological constituent in SMB, was found to attenuate the myocardial infarction pathological changes and improve heart function, and reduce macrophage infiltration, indicating the cardioprotective effects of Tanshinone IIA which might be attributed to its capacity for inhibiting inflammatory responses (Ren et al., 2010). Clinically, SMB has been applied to treat endometriosis and get satisfactory results. The present study was aimed to explore the effects of extracts of SMB (ESMB) on the serum levels of cancer antigen 125 (CA-125) and the levels of interleukin (IL)-13, IL-18 and tumor necrosis factor-alpha (TNF-alpha) in the peritoneal fluids of rat endometriosis models. These biomarkers for endometriosis have been found with clinical values (May et al., 2010; May et al., 2011). In the present study, danazol was used as the control intervention. Danazol produces an environment with high androgen and low estrogen levels, which leads to the atrophy of endometriotic implants (Selak et al., 2007).

Materials and methods

Chemicals and Materials

Tanshinone IIA (T4952) was purchased from Sigma Aldrich, UK. Salvianolic acid B, sodium danshensu were purchased from Carbosynth limited, UK. Structures are shown in Figure 1. Crude drug (approximately 0.5-1.0cm) and the commercial condensed powder of SMB were purchased from Beijing Tong Ren Tang Limited, China. Water and methanol of HPLC grade were purchased from Fisher Scientific UK. The liquid chromatographic system used was a Waters HPLC System (Waters, Milford, MA, USA) equipped with a 2996 photodiode-array (PDA) UV detector, Waters 626 Pump and

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Waters 600 Controller (LCD), Waters 717 plus Autosampler. The chromatographic separation of the three compounds was achieved by using a reversed-phase HPLC column (Agilent ZORBAX Rx-C18 2.1 x 150mm, 5 μ m).

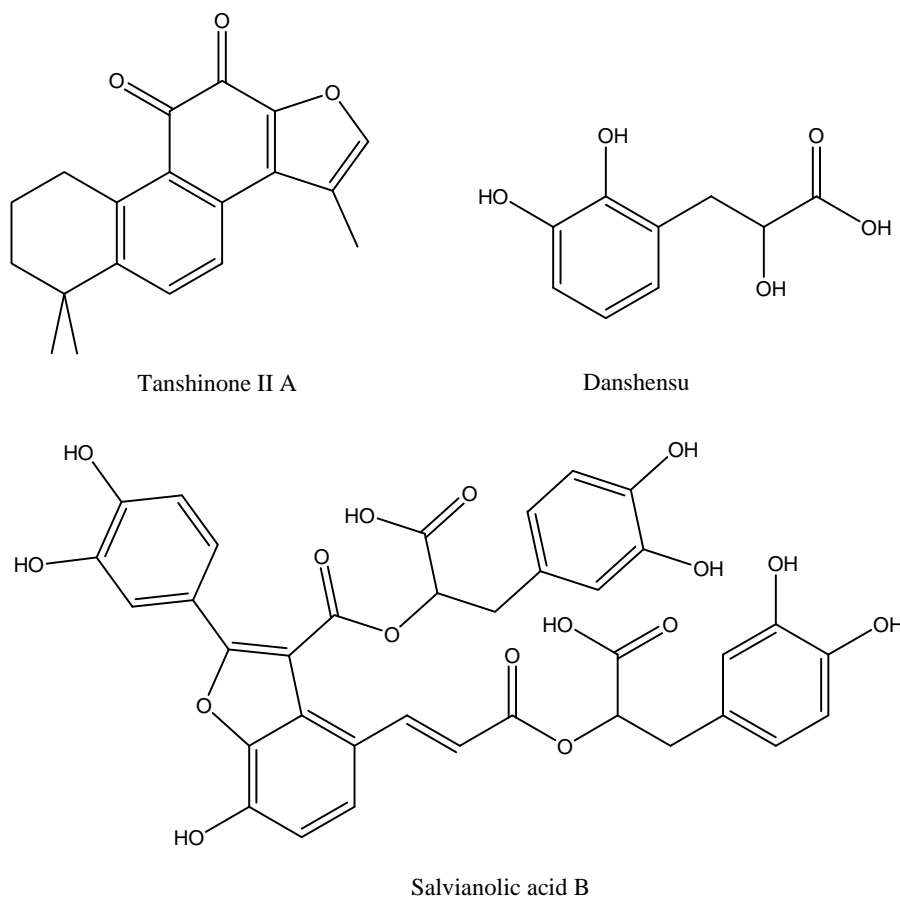


Figure 1. Structures of danshensu, salvia acid B and tanshinone II A in SMB

Initial Preparations of the sample extracted with conventional method

10g of crude drug of SMB was refluxed by Soxhlet extraction for 60min with 100 ml water. After filtration, the extraction procedure was repeated once with 80mL water. All the filtrates were combined and concentrated under reduced pressure and freeze dried. The flowchart of the extraction is shown in Figure 2.

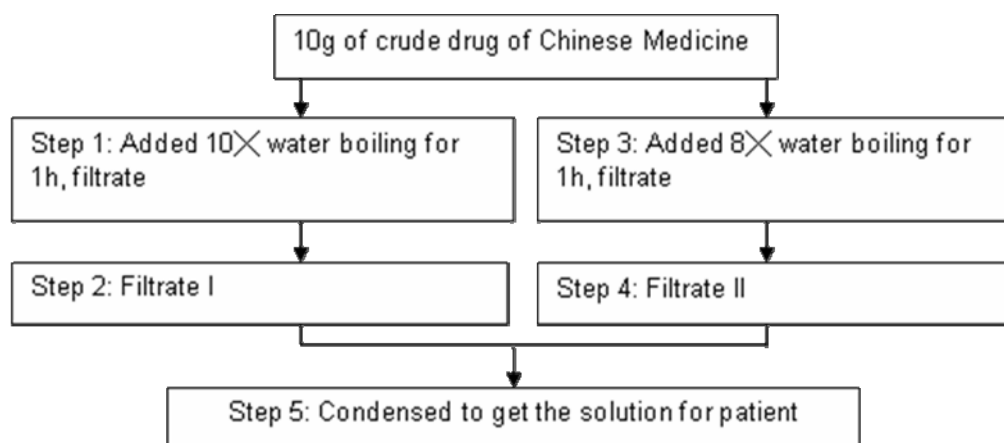


Figure 2: Flowchart of the conventional extraction method

Initial Preparations of the sample extracted with espresso coffee machine

An initial sample study consisted basically of a sample size study followed by a repeated extraction study. Plant materials were ground in a coffee grinder (De'longi, Italy). Studies were performed to compare different extraction times, and filtrate volumes for different sizes of Chinese medicine powders, in the same volume spoon. The mixed crude powders were shaken by Minor Test Sieve Shaker 230V/50HZ for 10 mins. And the powders past sieves test woven wire mesh brass BS 410 diameter (Endecotts, UK) with the aperture of 50 μ m, 100 μ m, 125 μ m, 500 μ m, 600 μ m, 710 μ m, 850 μ m and 1000 μ m, respectively. Compared with the conventional extraction method, the coffee machine extraction method only needed one step.

Different size of Chinese medicine powders were extracted by the espresso coffee machine (De'longi) for four times. Each time 80ml of filtrate was collected in the same volume beaker at ambient temperature. Then the filtrate was concentrated and freeze dried. The flowchart of the extraction is shown Figure 3.

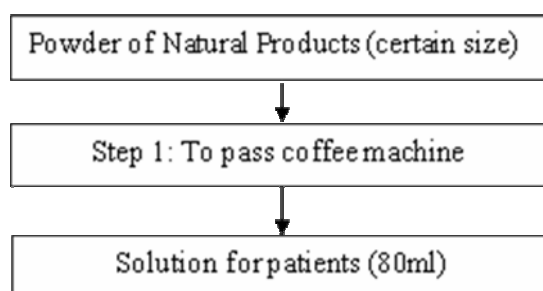


Figure 3: Flowchart of the coffee machine extraction method

Initial preparations of the commercial condensed powder of natural products

Hot water was added to the commercial condensed powder of SMB (Beijing Tong Ren Tang Limited, China) to make the solution for analysis.

Final Sample Preparation

The solution of natural product extracted with three different methods is freeze dried. The freeze dried powder was analyzed with HPLC method. Each freeze dried sample was accurately weighed and dissolved with water and filtered through a membrane filter (0.45µm) before HPLC analysis.

HPLC analysis for SMB

The gradient elution for HPLC analysis consisted of two solvent compositions: 0.1% acetic acid in water (solvent A) and methanol (solvent B). Gradient elution was carried out according to the following program: solvent B was increased from 5 to 30% in the first 25 min, then increased to 35% till 40min, increased to 75% till 60min and then increased to 85% at 70 min. The flow rate was 0.25µl/min. The sample injection volume was 10µl. The column and auto-sampler were set at ambient temperature. The eluent was monitored by a UV detector at the wavelength of 280 nm for all the studied compounds.

Preparation of standard solutions and the calibration curves for SMB

A standard stock solution 730.0 µg/ml of salvianolic acid B and 470.0 µg/ml of sodium danshensu were prepared separately in 50% methanol. A standard stock solution 280.0µg/ml of tanshinone IIA was prepared separately in methanol. Standard working solution of salvianolic acid B, tanshinone IIA, and sodium danshensu were prepared by diluting their original stock solutions with 50% methanol for salvianolic acid B and sodium danshensu, and methanol for tanshinone IIA, to reach the final concentrations of 73.0, 47.0, and 28.0 µg/ml, respectively. The mixed standard solution was prepared with 200ml of salvianolic acid B, sodium danshensu, and methanol for tanshinone IIA mixed together with the final content of 15.0, 16.0, and 9.3 µg/ml of salvianolic acid B, sodium danshensu, and methanol for tanshinone II A respectively. Working standard solutions and the quality control sample solutions at high, medium and low concentrations were prepared by diluting the standard stock solutions with 50% methanol for sodium danshensu and salvianolic acid B, and methanol for tanshinone IIA. These solutions were all kept in brown glass bottles and stored at -20 °C. Calibration curves were constructed by plotting the peak-area ratio of each analyte standard versus analyte concentration. The studied concentration ranges were 0.73–73.0µg/ml for salvianolic acid B and 0.48–48.0µg/ml for danshensu and 2.8–28.0µg/ml for tanshinone IIA.

Validation of method

Validation of the HPLC method was performed by determining the intra-day, inter-day accuracy and precision. The quality control samples were analyzed in a set of five on a single assay day to determine intra-day precision and accuracy, and analyzed in duplicate on each of three separate days to determine inter-day precision and accuracy. The quality control samples at low, medium and high concentrations (salvianolic acid B: 18.3, 36.5, 73 µg/ml; danshensu: 11.8, 23.5, 47.0µg /ml; tanshinone IIA: 7.0, 14.0, 28.0µg/ml) were used. The limit of detection was defined as the lowest concentration of the analyst resulting in a signal-tonoise ratio of 3:1.

Establishment of the model rats with endometriosis

Forty female Sprague-Dawley (SD) rats with body weight of 140 ±20 g were purchased from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The animals were kept in a room under a 12h light–12h dark cycle and environmentally controlled conditions of 22±2°C. The whole protocol of the study was conducted based on the National

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Research Council's protocol for the care and use of laboratory animals and was approved by the Institutional Review Board. Among the 40 rats, 10 rats were randomly taken as the sham-operation group (Sham group). The other 30 rats were used to establish the model rats with endometriosis. The method of operational transplantation was used in the present research to establish the model rats with endometriosis. Each rat was injected with 0.2mg of diethylstilbestrol to stimulate estrus. Then 20% urethane (1.5g/kg) was injected intra-abdominally. The abdominal fur was shaved and the skin disinfected. The abdominal cavity was opened and the uterus was separated away from the right ovary by 0.5cm, and 2-cm long section of the uterus removed. Then the endometrium was separated and divided into three parts and the uterine branch, left ovary and parietal peritoneum were respectively sutured. Finally the abdominal cavity was closed and gentamycin sulfate (0.1ml) was injected into each rat for 3 days after finishing the above operation. Four weeks after the model rats were made, the 30 model rats were randomly divided into three groups (n=10 in each group). For each rat in the Sham group, after the abdominal fur was shaved and the skin disinfected, the abdominal cavity was opened and then closed. The Sham group was used to insure aseptic conditions.

Group and administration

The 40 rats were randomly divided into four groups: ESMB group (the rats were orally administrated the extracts of SMB at 150 mg/kg once daily for 56 consecutive days), Danazol group (the rats were orally administrated danazol at 36 mg/kg once daily for 56 consecutive days), model control group (Model group, the rats were orally administrated saline at 8ml/kg once daily for 56 consecutive days), and Sham group (the rats were orally administrated saline at 8ml/kg once daily for 56 consecutive days). After all the treatment ended, the rats were sacrificed and the samples of peritoneal fluids and serum were taken. The peritoneal fluid samples of the rats were centrifuged at 12,000 rpm for 10 min at 4 °C. Then, the supernatants were collected, aliquoted, and stored frozen at -80 °C until used for further evaluation. The serum levels of CA-125 and the levels of IL-13, IL-18 and TNF-alpha in the peritoneal fluids were detected using enzyme-linked immune-sorbent assay (ELISA) as directed by the manufacturer (RUIQI Bio Co. Ltd, Shanghai, China). Danazol was provided by Lianhua Medicine Company (Jiangsu, China).

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS 19.0 for Windows). All the data were found normally distributed using the Kolmogorov-Smirnov (KS) test. The comparisons among different groups were performed with one-way analysis of variance (ANOVA) and multiple comparison tests were conducted with Bonferroni correction procedure. For all the hypothesis tests, significance level was set at $P=0.05$ and two-tailed tests were used.

Results

Content determination of salvia Acid B, tanshenone II A and danshensu in SMB

The calibration equation of salvia acid B, tanshenone II A and danshensu is $Y=1553098.34X-80999.50$ ($R^2=0.9997$); $Y=20953001.7X-191279.55$ ($R^2=0.9993$) and $Y=499565.11X+141436.50$ ($R^2=0.9994$). Figure 8 shows the concentration of salvia Acid B, tanshenone II A and danshensu in ESMB extracted with different methods. Figure 8 presents that the concentration order from high to low of danshensu is 600-710 μ m (extracted by coffee machine) > 500-600 μ m (extracted by coffee machine) > 125-500 μ m (extracted by coffee machine) > conventional boiling method > condensed powder. The concentration order from high to low of salvia acid B is conventional boiling method > 600-710 μ m (extracted by

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coffee machine) > 125-500 μ m (extracted by coffee machine) > 500-600 μ m (extracted by coffee machine) > condensed powder.

The concentration order from high to low of tanshentone II A is 600-710 μ m (extracted by coffee machine) > 125-500 μ m (extracted by coffee machine) > 500-600 μ m (extracted by coffee machine) > conventional boiling method > condensed powder. ESMB with the powder size between 600-710 μ m extracted with espresso coffee machine has higher concentration of salvianolic acid B, tanshinone IIA and sodium danshensu. At the same time, the coffee machine extraction method requires less time and less sample mass than the conventional method. The HPLC profile of ESMB with the powder size between 600 μ m -710 μ m is shown in Figure 4. The HPLC profile of commercial condensed SMB powder is shown in Figure 5.

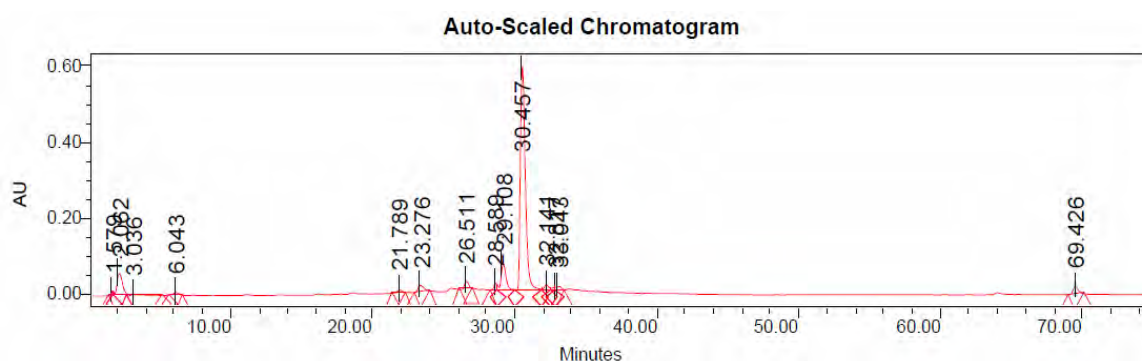


Figure 4: HPLC profile of ESMB powders (600 μ m - 710 μ m) extracted by coffee machine.

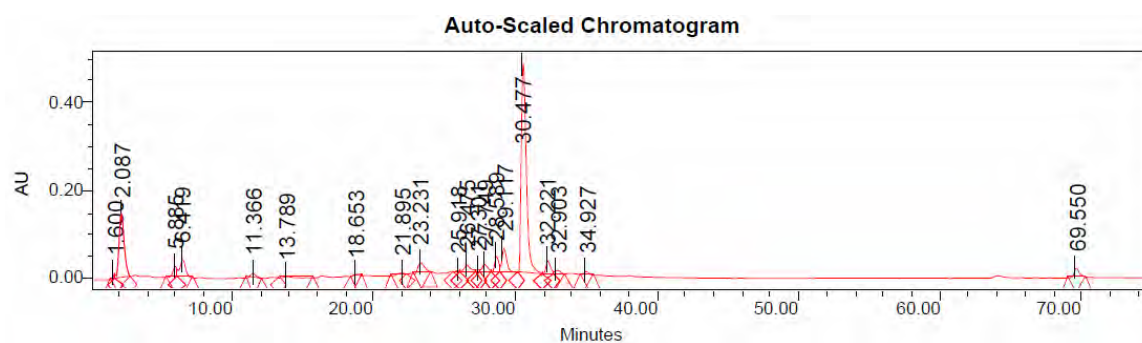


Figure 5: HPLC profile of commercial condensed SMB powders

The serum levels of CA125

As shown in Figure 6, the serum levels of CA-125 in ESMB group, Danazol group and Sham group were significantly lower than those of the Model group ($P < 0.05$). The serum levels of CA-125 in ESMB group and Danazol group were significantly higher than those of Sham group ($P < 0.05$). The serum level of CA-125 in ESMB group was significantly higher than that of Danazol group ($P < 0.05$).

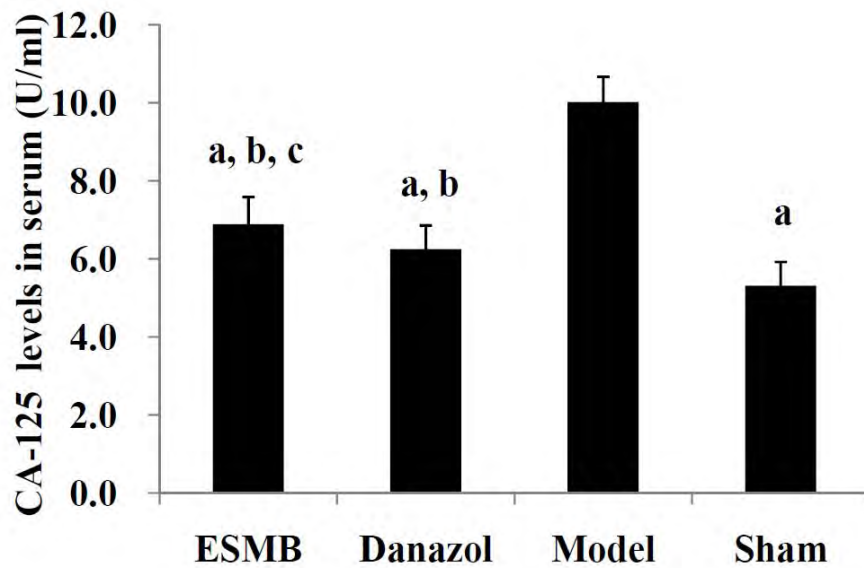


Figure 6: The serum levels of cancer antigen 125 (CA-125)

Data were shown as mean \pm SD. (N=10 in each group). The significant difference was set at ^a $P < 0.05$, compared with the Model group; ^b $P < 0.05$, compared with the Sham group; ^c $P < 0.05$, compared with the Danazol group.

The levels of IL-13, IL-18 and TNF-alpha in the peritoneal fluids

As shown in Figure 7, the levels of IL-13 in the peritoneal fluids of ESMB group, Danazol group and Sham group were significantly higher than those of the Model group ($P < 0.05$). The levels of IL-13 in the peritoneal fluids of ESMB group and Danazol group were significantly lower than those of Sham group ($P < 0.05$), and there was no marked difference between ESMB group and Danazol group ($P > 0.05$).

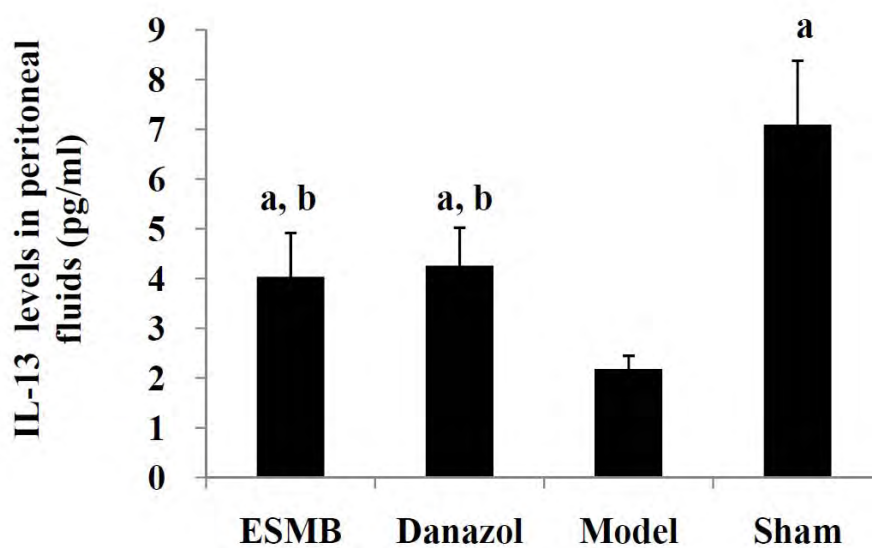


Figure 7: The levels of interleukin (IL)-13 in the peritoneal fluids

Data were shown as mean \pm SD. (N=10 in each group). The significant difference was set at ^a $P < 0.05$, compared with the

As shown in Figure 8, the levels of IL-18 in the peritoneal fluids of ESMB group, Danazol group and Sham group were significantly lower than those of the Model group ($P < 0.05$). The levels of IL-18 in the peritoneal fluids of ESMB group and Danazol group were significantly higher than those of Sham group ($P < 0.05$), and there was no marked difference between ESMB group and Danazol group ($P > 0.05$).

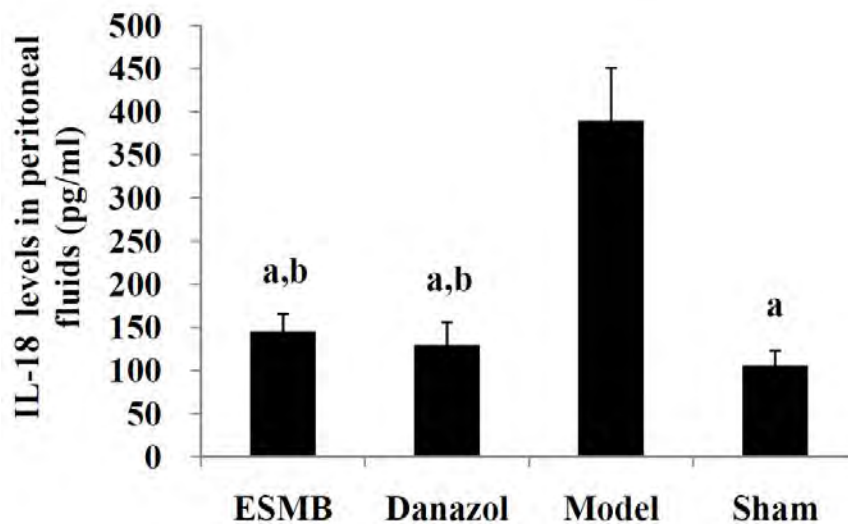


Figure 8: The levels of IL-18 in the peritoneal fluids

Data were shown as mean \pm SD. (N=10 in each group). The significant difference was set at ^a $P < 0.05$, compared with the Model group; ^b $P < 0.05$, compared with the Sham group; ^c $P < 0.05$, compared with the Danazol group.

As shown in Figure 9, the levels of TNF-alpha in the peritoneal fluids of ESMB group, Danazol group and Sham group were significantly lower than those of the Model group ($P < 0.05$). The levels of TNF-alpha in the peritoneal fluids of ESMB group and Danazol group were significantly higher than those of Sham group ($P < 0.05$), and no marked difference existed between ESMB group and Danazol group ($P > 0.05$).

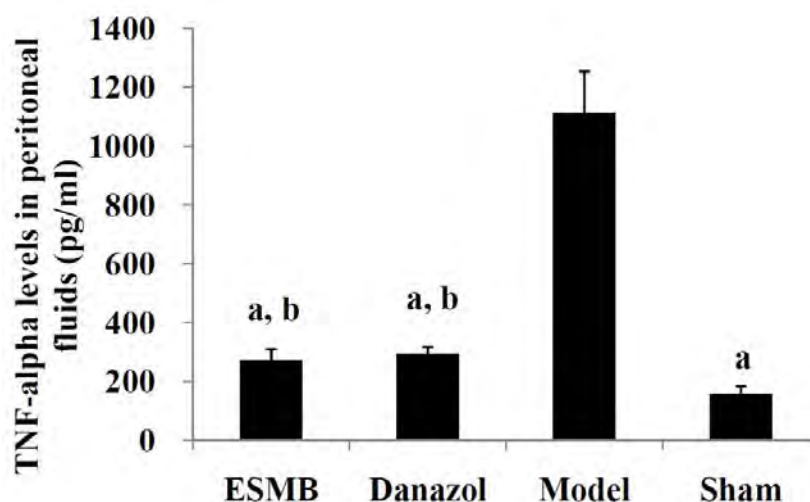


Figure 9: The levels of tumor necrosis factor-alpha (TNF-alpha) in peritoneal fluids

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<http://dx.doi.org/10.4314/ajtcam.v9i3.2>

Data were shown as mean \pm SD. (N=10 in each group). The significant difference was set at ^a $P < 0.05$, compared with the Model group; ^b $P < 0.05$, compared with the Sham group; ^c $P < 0.05$, compared with the Danazol group.

Discussion

In the present study, CA125, IL-13, IL-18 and TNF-alpha were chosen to reflect the effects of ESMB on the model rats with endometriosis. Serum CA-125 measurement is now a consolidated method for diagnosing endometriosis, and the serum CA-125 values were found significantly elevated in patients with ovarian and mixed endometriosis lesions (Patrelli et al., 2011). The levels of IL-18 in peritoneal fluids were found markedly higher in women with peritoneal, minimal- to mild-stage endometriosis than in controls (Arici et al., 2003). The levels of TNF-alpha in peritoneal fluids was demonstrated as a biomarker to discriminate between patients with endometriosis and those without (Bedaiwy et al., 2002). We found that ESMB markedly decreased the serum levels of CA-125 and the levels of IL-18 and TNF-alpha in the peritoneal fluids and significantly increased the levels of IL-13 in the peritoneal fluids.

Tanshinone IIA, a major component extracted from SMB can improve blood circulation and treats chronic hepatitis and hepatic fibrosis. Tanshinone IIA was found to induce apoptosis as demonstrated by DNA fragmentation, poly(ADP-ribose) polymerase and caspase-3 cleavage, increase Bax/Bcl-2 protein ratio, and depolarize of mitochondrial membranes to facilitate cytochrome c release into the cytosol (Che et al., 2010). Tanshinone IIA can reduce the increase of caspase-3 activity induced by the amyloid beta-peptide (A β), which strongly suggested that tanshinone IIA may be effective in treating Alzheimer disease associated with oxidative stress (Liu et al., 2010). A recent study showed that tanshinone IIA strongly inhibited the growth of cervical cancer cells through interfering in the process of microtubule assembly, which led to G(2)/M phase arrest and sequent apoptosis (Pan et al., 2010). SMB and tanshinone IIA can be used to induce Schwann cell proliferation, and in vivo results potentially suggested that SMB and tanshinone IIA might enhance neuron regeneration (Shen et al., 2011). Signal transducer and activator of transcription 3 (STAT3) is usually constitutively activated in a variety of malignancies, and STAT3 may be a promising target for treatment of tumor cells. Tanshinone IIA may serve as an effective adjunctive reagent in the treatment of glioma for its targeting of constitutive STAT3 signaling (Tang et al., 2010). Tanshinone IIA were also found to induce apoptosis involving mitochondria intrinsic caspase activation cascade and an inhibition of the phosphoinositide 3-kinase /AKT survival pathway (Won et al., 2010). In a study designated to evaluate the possible neuroprotective effects of tanshinone IIA on hydrogen peroxide-induced oxidative stress in rats, the researchers demonstrated that tanshinone IIA might serve as a novel promising therapeutic agent for oxidative stress injury in neurodegenerative diseases (Wang et al., 2011). Tanshinone IIA has multiple effects in the inhibition of the migration of human aortic smooth muscle cell and may offer a therapeutic approach to block the process (Jin et al., 2008). It has limited brain penetration through the blood-brain barrier owing to the contribution of P-gP and multidrug-resistance-associated protein (Mrp1/2) (Chen et al., 2007).

A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed and validated for the determination and pharmacokinetics of danshensu in rat plasma samples using ferulic acid as internal standard (IS) and the validated LC/MS/MS method was applied to a pharmacokinetic study in which the extracts of SMB (ESMB, containing 40 mg/g danshensu) was administered orally to rats at a single dose of 200 mg/kg in 2% water (Li et al., 2008). A dynamic continuous ultrasound-assisted extraction with high intensity ultrasonic probe (CUAE-HIUP) combined with solid-phase extraction (SPE) for preconcentration and clean-up of the extract prior to high-performance liquid chromatography (HPLC) determination of the main biological active ingredients, sodium Danshensu and four tanshinones (dihydrotanshinone I, tanshinone I, cryptotanshinone and tanshinone IIA) from root of SMB has also been developed in a recent study. and the method was successfully applied to the determination of the five biological active ingredients in root of SMB and SMB-containing pharmaceutical formulations (Yang et al., 2007).

In the present study, three extraction methods for SMB were compared, which are the samples extracted with conventional method, the sample extracted with espresso coffee machine and the commercial condensed powder of natural products. The extraction efficiency of the ESMB samples range from 600µm to 710µm is the highest. We determined tanshinone IIA, salvianolic acid B and danshensu in the ESMB of different extraction methods. Further studies are needed to be conducted on the other potential mechanisms involved in ESMB relieving endometriosis.

Acknowledgements

Dr. Jue Zhou was funded by Research Fund of Zhejiang Gongshang University (1110XJ2011023).

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