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Therapeutic effects of standardized *Vitex negundo* seeds extract on complete Freund's adjuvant induced arthritis in rats

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ABSTRACT

The seeds of *Vitex negundo* L. (Verbenaceae) have been commonly used as a folk remedy for the treatment of rheumatism and joint inflammation in Traditional Chinese Medicine. This study aimed to evaluate the anti-arthritic activity of the extract of *V. negundo* seeds (EVNS) using Freund's complete adjuvant (CFA) induced arthritis (AA) in rat model. As a result, EVNS, with abundant phenylnaphthalene-type lignans, significantly inhibited the paw edema, decreased the arthritis score and spleen index, and reversed the weight loss of CFA-injected rats. Histopathological studies showed a marked decrease of synovial inflammatory infiltration and synovial lining hyperplasia in the joints of EVNS-treated animals. The remarkable decrement of serum inflammatory factors (TNF-α, IL-1β and IL-6) were observed in EVNS-treated rats, whereas, IL-10, an anti-inflammatory cytokine, was found to be significantly increased by EVNS. The expressions of COX-2 and 5-LOX in PBMC were also inhibited by administration of EVNS. Our results demonstrated that *V. negundo* seeds possessed potential therapeutic effect on adjuvant induced arthritis in rats by decreasing the levels of TNF-α, IL-1β and IL-6 and increasing that of IL-10 in serum as well as down-regulating the levels of COX-2 and 5-LOX, and therefore may be an effective cure for the treatment of human rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is one of the most chronic destructive diseases to human health, which is also called "immortal cancer", characterized by inflammatory cell infiltration and proliferation of synovial tissue, accompanied by bone destruction (Imboden 2009; Pincus and Callahan 1993). It can rapidly progress into multisystem inflammation with irreversible joint damage thus causing premature mortality, disability and compromised quality of life in the industrialized and developing world (Silman and Hochberg 2001; Brooks 2006).

Nowadays, disease-modifying antirheumatic drugs (DMARDs) supplemented with non-steroidal anti-inflammatory drugs (NSAIDs), steroid hormone and biologics (TNF-α antibody and the decoy TNF-α receptor, etc.) remains the major strategy in the treatment of RA (Silman and Hochberg 2001; Scott et al. 1998). According to the guidelines of the American College of Rheumatology, newly diagnosed RA patients were strongly recommended to begin treatment with NSAIDs for relieving nociceptive pain and controlling inflammation, with combined use of DMARDs for reducing disease activity, preventing joint deformity and improving joint function (Doan and Massarotti 2005). However, administration of these drugs is associated with severe adverse effects, including gastrointestinal lesions, cardiovascular complications, reproductive toxicity, and etc. (Couzin 2004; Kremers et al. 2004). Therefore, more and more attention has been paid to plant-derived anti-RA drugs with high efficacy and few side effects. Recent investigations have estimated that 60–90% of RA patients are very likely to use botanicals (Wang et al. 2012). This growing interest in alternative medical practices clearly indicates the need for more safe and effective anti-RA botanicals used in the traditional medicine.

Vitex negundo L. (Verbenaceae), an important medicinal plant in the *Vitex* species, has been used as reputed herbal medicine with versatile pharmacological activities in China, India, Japan,

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Indonesia, East Africa and South America (Zheng et al. 2009a). The seeds of this plant are commonly used as a condiment for edible purpose (Kunkel 1984) and have been widely used in folk medicine for anti-inflammatory (Zheng et al. 2009a, 2010; Chawla et al. 1992), analgesic (Zheng et al. 2009b), and antioxidant purposes (Ono et al. 2004), containing abundance of bioactive lignans (Zheng et al. 2009a; Ono et al. 2004), terpenoids (Chawla et al. 1992; Zheng et al. 2010; Ono et al. 2004) and flavonoids (Chawla et al. 1991; Bhargava 1989). In Traditional Chinese Medicine, *V. negundo* seeds were commonly used for a series of inflammation related disorders, such as rheumatism, chronic bronchitis, chronic gastritis and colitis (Vimal et al. 2011). In Ayurvedic Medicine, *V. negundo* seeds also found a good reputation for the treatment of rheumatoid arthritis (Mohiuddin et al. 1977). In our previous studies, we also demonstrated the significant antinociceptive and antiinflammatory activities of the extract of *V. negundo* seeds (EVNS) in mice in a dose-dependent manner (Zheng et al. 2009b). The traditional use of *V. negundo* seeds as an anti-inflammatory agent, together with modern pharmacological studies, suggested it has potential therapeutic effects in the treatment of RA, although no previous studies have focused on the *in vivo* anti-RA activity of this medicinal seed. Therefore, the present study was conducted to investigate the efficacy of the extract of *V. negundo* seeds (EVNS) as anti-arthritic agents and explore its potential mechanism on adjuvant-induced arthritis (AA) in rats.

Materials and methods

Chemicals and solvents

Complete Freund's adjuvant (CFA) and Histopaque 1083 were purchased from Sigma Chemical Co., USA. Methotrexate was bought from Shanghai Sine Pharmaceutical Co., Ltd., China. ELISA kits of TNF-α, IL-1β, IL-6, and IL-10 were purchased from R&D system, USA, while COX-2 and 5-LOX were bought from MyBioSource, USA. All the other chemicals and biochemical used were of the highest grade available.

Plant material

The seeds of *V. negundo* (Chinese name 'Huang-Jing-Zi') were obtained from Wanglang National Nature Reserve, Sichuan Province, and were identified by Professor Lu-ping Qin, Second Military Medical University. A voucher specimen (#2006-168) has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University.

Preparation of extract

The preparation of the extract of *V. negundo* seeds (EVNS) was conducted according to the method described by Zheng et al. (2009b). Briefly, the air-dried and powdered seeds were extracted with 80% EtOH three times (3×) for 2 h each time. After removal of the solvent under reduced pressure, the residue was evaporated to dryness. The dry residue was suspended in water with 1% w/v sodium carboxyl methyl cellulose (Na-CMC) for pharmacological studies. The doses employed are expressed as mg of the dried extract per kg body weight.

HPLC analysis of the extract of V. negundo seeds (EVNS)

The extract of *V. negundo* seeds (EVNS) was dissolved in 50% methanol prior to HPLC analysis. This was performed on an Agilent-1100 system with a Zorbax Extend-C18 chromatographic column (250 mm × 4.6 mm, 5 μm) at 30 °C with a H_2O (+0.1% HCOOH) (A)/acetonitrile (B) gradient, a sample injection volume of 20 μl, flow rate of 0.8 ml/min, and a detection wavelength 254 nm. Samples were analyzed by using a gradient program as follows: run was commenced with 8% B, linear gradient to 15% B within 14 min, followed by linear gradient to 25% B in 21 min, and finally linear gradient to 55% B in 35 min.

Animals

Male Wistar rats weighing 160–180 g body weight were procured from the Experimental Animal Center of Second Military Medical University (Shanghai, China). They were kept in an environment with controlled temperature (24–26 °C) and photoperiod (12:12 h) light–dark cycle. Animals were given standard commercial rat chow and water *ad libitum* and processed following the internationally approved ethical guideline of the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were carried out with the approval of the Animal Experimentation Ethics Committee of the Second Military Medical University (approval number: 20130214).

Adjuvant induction

Complete Freund's adjuvant (CFA, sigma) was prepared by suspending heat-killed BCG in liquid paraffin at 10 mg/ml. Each rat, except the normal control group, was injected intradermally with 0.1 ml of Freund's complete adjuvant (Sigma product) into the left hind metatarsal footpad of rat for induced inflammation (Sindhu et al. 2011). The animals were divided into five groups of six animals in each as follows:

- Group I – Normal control
- Group II – Rats with adjuvant-induced arthritis (AA)
- Group III – AA + methotrexate (3 mg/kg, twice a week, orally by gastric intubation)
- Group IV – AA + EVNS (340 mg/kg/day, orally by gastric intubation)
- Group V – AA + EVNS (85 mg/kg/day, orally by gastric intubation).

The normal group and AA model group were given an equal volume of the vehicle (CMC-Na) at the same time. During the course of the experiment, the body weight of rats was measured every 7 days. And the rats were assessed every three days for signs of arthritis between days 1 and 28. Each paw was recorded on an ordinal scale as follows: 0 = unaffected, 1 = 1 type of joint affected, 2 = 2 types of joints affected, 3 = 3 types of joints affected, 4 = 3 types of joints affected and maximal erythema and swelling (Kokkola et al. 2003). The maximum arthritic score per rat was set at 8 (4 points × 2 hind paws). The rat paw edema was measured on days 0, 7, 14 and 21 expressed as the foot-breadth of ankle by vernier caliper. On the 28th day, the rats were sacrificed by decapitation. Thymus and spleen were dissected out, washed in ice-cold saline, patted dry and weighed (Sundaram et al. 2011). The indices of thymus and spleen were expressed as the ratio (mg/g) of thymus and spleen wet weight *versus* body weight, respectively (Zhang et al. 2004).

Index of thymus and spleen assay

At day 28 after immunization, the rats were sacrificed via anesthesia (pentobarbital sodium, 40 mg/kg, i.p.). The thymus and spleen were then promptly removed and weighed. The index of thymus and spleen were expressed as the ratio of thymus and spleen wet weight *versus* body weight (mg/g), respectively (Zhang et al. 2004).

Isolation of peripheral blood mononuclear cells (PBMC)

The experiment was carried out according to the modified method of Radhika et al. (2007). Briefly, 3 ml Histopaque 1083

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