Journal of Ethnopharmacology 153 (2014) 928–932

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Ethnopharmacological communication

Anti-stress effect of astragaloside IV in immobilized mice

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ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
21 March 2014
Accepted 30 March 2014
Available online 5 April 2014

Keywords:
Astragalus membranaceus
Astragaloside IV
Anxiety
Inflammation
Immobilization stress

ABSTRACT

Ethnopharmacological relevance: Astragaloside IV, a major component extracted from the roots of *Astragalus membranaceus* (AM), possesses anti-inflammatory, anti-oxidative, anti-fibrotic, anti-infarction and immunoregulatory effects. To clarify anti-stress effect of AM, anxiolytic and anti-inflammatory effects of 80% ethanol extract of AM and astragaloside IV were investigated in immobilization stress model.
Materials and methods: The mice were orally administered with AM (50, 200, and 500 mg/kg), astragaloside IV (5, 10, and 20 mg/kg) and buspirone, a positive drug, 1 h before immobilization treated for 2 h. For anxiolytic activity assay, EPM test was performed in mice. For anti-inflammatory activity assay, serum levels of corticosterone, IL-6 and TNF-α were measured using ELISA kits.
Results: AM extract and astragaloside IV increased dose-dependently time spent on open arms and open arm entries in the EPM test. Anxiolytic effects of AM extract (500 mg/kg) and astragaloside IV (20 mg/kg) were comparable to those of buspirone (1 mg/kg). Their anxiolytic effects were blocked by WAY-100635 (0.5 mg/kg, *i.p.*), a 5-HT_{1A} receptor antagonist ($p < 0.01$), but not by flumazenil (3 mg/kg, *i.p.*) and bicuculline (0.5 mg/kg, *i.p.*), $GABA_A$ receptor antagonists. AM extract and astragaloside IV also reduced serum levels of corticosterone, IL-6 and TNF-α dose-dependently.
Conclusions: AM, particularly astragaloside IV, may ameliorate immobilized stress-induced anxiety and inflammation.

1. Introduction

Stress is an unavoidable part of life in modern society and causes alterations of the immune, neuroendocrine, and sympathetic nervous system, which could lead to interference of host defenses (Dugue et al., 1993; Kiecolt-Glaser et al., 2003; Koolhaas et al., 2011). The failure to cope with stress is closely associated with the occurrence and progression of disorders such as depression, anxiety, cancer, cardiovascular disturbance and post-traumatic stress disorders (Strekalova et al., 2005). Anxiety is an unpleasant state of inner turmoil, often accompanied by nervous behaviors and caused by many kinds of stress and affects one-eighth of the world's population (Eisenberg et al., 1998). In the field of anxiety, the elevated plus-maze (EPM) has become one of the most popular animal models. The anxiety-like behavior is potentiated in the EPM by the prior exposure to a variety of stressors, such as immobilization, social defeat, forced swim and inescapable footshock (Korte and De Boer, 2003). Stress-induced activation of the sympathetic-adrenal medulla and the hypothalamo-pituitary-adrenal (HPA) axis simulates secretion of glucocorticoid, noradrenaline, and adrenaline, which are capable of modulating immune cells and further modulating cytokine production (Haddad et al., 2002; Padgett and Glaser, 2003; Sekiyama et al., 2006). Among cytokines, proinflammatory cytokines, tumor necrosis factor (TNF)-α and interlukin (IL)-6 are up-regulated (Liu et al., 2007).

Astragalus membranaceus Bunge (AM, Leguminosae) has been used in traditional Chinese medicine to enhance the body's defense system, reinforce skin, drain abscess, generate tissue and reduce a variety of stresses (Cho and Leung, 2007; Kusum et al., 2004; Liu et al., 2012; Yin et al., 2004; Zhang et al., 2012). AM contains isoflavones, saponins and polysaccharides (Li et al., 2007; Ma et al., 2002). Among these constituents, astragaloside IV, a representative constituent of AM, has been reported to exhibit protective effect for myocardinal ischemia, nociception, herpes virus, coxsackie virus, diabetic nephropathy and regulate immune function to inhibit the TNF-α and IL-4 (Wu and Chen, 2004; Yesilada et al., 2005; Yin et al., 2004). Recently AM-contained formula is reported to protect stress-associated oxidative stress damage (Kim et al., 2012; Lee et al., 2012). Nevertheless, the anti-stress effects of AM and its constituents have not been studied. Therefore, we investigated anxiolytic and anti-inflammatory effects

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http://dx.doi.org/10.1016/j.jep.2014.03.068
0378-8741/

of 80% ethanol extract of AM and its representative constituent, astragaloside IV, in immobilization stress-induced mice.

2. Materials and method

2.1. Material

Astragaloside IV, bicuculline, buspirone, dexamethasone, flumazenil and WAY-100635 were purchased from Sigma (St. Louis, MO, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcam (Cambridge, MA, U.S.A.). Acetonitrile and water for HPLC analysis were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

2.2. Preparation of extract and its HPLC analysis

The roots of *Astragalus membranaceus* (AM) were collected in Jeongseon province, Korea, in November 2012, and identified by Nam-Jae Kim, a coauthor of the present study. A voucher specimen (KHUP1210011) was deposited at College of Pharmacy, Kyung Hee University. The air-dried roots (200 g) were extracted twice with 2 L of 80% ethanol at 100 °C for 2 h and the extract was concentrated with a rotary evaporator and freeze-dried to yield 17.8% (w/w).

Phytochemical characteristics of AM extract were identified by high-performance liquid chromatography (HPLC) analysis. The system was equipped with Alliance 2690 Separation Module, a Waters 996 Photodiode Array Detector and a Millenium^{32} Chromatography Manager Version 3.2. The chromatographic separation was performed using a Nucleosil C_{18} column (4.0 mm × 250 mm I. D., Waters) and the column temperature was kept at 25 °C. The mobile phase was 40% acetonitrile, the flow rate was 1.0 ml/min and the wavelength was set at 203 nm. Astragaloside IV content of the AM extract was 0.51%.

2.3. Animals

Male ICR mice (7 weeks-old, 24–28 g) were purchased from Samtako Biokorea (Seoul, Korea) and acclimated for 1 week before use. All animals were maintained under a constant temperature (24 ± 2 °C) and humidity (60 ± 10%) with an alternating 12 h light–dark cycle. They were fed on standard laboratory chow (Samyang Co., Seoul, Korea) with tap water ad libitum. Each group consisted of 7 mice in all experiment. Mice were randomly divided in 9 groups: normal control (non-immobilization and vehicle), control (immobilization and vehicle), AM (immobilization and 50, 200, or 500 mg/kg of AM extract), AS (immobilization and 5, 10, or 20 mg/kg of astragaloside IV) and positive control (immobilization and 1 mg/kg of buspirone or dexamethasone). Each sample was orally administered to mice 1 h prior to immobilization. Buspirone was intraperitoneally administered 30 min prior to immobilization.

For an anxiolytic antagonism study, after treated with AM (200 mg/kg, *p.o.*) and astragaloside IV (20 mg/kg, *p.o.*), mice were administered with flumazenil (3 mg/kg, *i.p.*), bicuculline (0.5 mg/kg *i.p.*) or WAY-100635 (0.5 mg/kg *i.p.*) 30 min prior to immobilization.

All experiments were performed in accordance with the National Institutes of Health and Kyung Hee University guides for Laboratory Animals Care and Usage. The protocol was approved by the Institutional Animal Care and Use Committee of the Kyung Hee Medical Center and Kyung Hee University.

2.4. Immobilized stressors

Immobilized stress was performed with slight modification according to the previous method (Satoh et al., 2006). It was accomplished by placing the mouse vertically in 50 ml conical tube (3 cm in diameter and 10 cm in length) and gauze was inserted to prevent forward and backward movements and limit side-to-side mobility. A 0.3-cm-diameter hole was made on the center of tube for mouse to breathe and mice were immobilized in the tubes for 2 h.

2.5. EPM test

The EPM test was performed as previously described (Grundmann et al., 2007). The plus-maze apparatus consists of two open arms (30 × 7 cm) and two enclosed arm (30 × 7 cm) with 20 cm high walls, extending from a central platform (7 × 7 cm). The maze made of the black plexiglass was raised 50 cm above the floor in a dimly lit room (20 lux) and a video camera was suspended above the maze to record the mouse's movements.

Each mouse was individually placed at the center of EPM, its head facing the open arm and tested individually for 5 min. An arm entry was defined as entry of all four paws into an arm and the behavior of the mouse was recorded as the time spent on open or closed arms and the number of entries into open or closed arms. And then, the percentage of time spent in open arms [(time spent in open arms/time spent in open and closed arms) × 100] and of open arm entries [(open arm entries/entries of open and closed arm) × 100] was calculated for each animal. After each trial, the maze was cleaned with 70% ethanol to remove any residue and odors.

2.6. ELISA assay

Serum levels of corticosterone, IL-6 and TNF-α were measured using commercial ELISA kits. Assays were performed by following the instruction provided by the manufacturers.

After immobilizing for 2 h, mice were sacrificed. The blood samples were collected from carotid artery and centrifuged at 3000 rpm for 5 min at 4 °C. Serum was separated and frozen at −70 °C until assayed.

2.7. Statistical analysis

All data are expressed as the mean ± S.E.M. The significant difference was analyzed by one way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple comparisons. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of AM extract and astragaloside IV on immobilization-induced anxiety-like behaviors in the EPM test

Immobilization stress significantly decreased time spent in open arms (OT) and open arm entries (OE) in mice (Fig. 1). Treatment with AM extract or astragaloside IV in immobilization stress-treated mice attenuated the decrease in OT and OE dose-dependently. AM extract (200 and 500 mg/kg) and astragaloside IV (10 and 20 mg/kg) showed anxiolytic-like effect ($p < 0.001$). Their anxiolytic effects were comparable to those of buspirone (1 mg/kg), an anxiolytic agent, which significantly increased OT and OE ($p < 0.001$).

3.2. Effect of flumazenil, bicuculline and WAY-100635 on the anxiolytic activity of AM extract and astragaloside IV in the EPM test

In order to understand their anxiolyic mechanism, we examined the effect of AM extract (200 mg/kg, *p.o.*) and astragaloside IV (20 mg/kg, *p.o.*) with or without flumazenil (3 mg/kg, *i.p.*),