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Kaempferol from the leaves of *Apocynum venetum* possesses anxiolytic activities in the elevated plus maze test in mice

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Abstract

The present work evaluated the anxiolytic activity of an aqueous extract of *Apocynum venetum* L. (Apocynaceae) and bioguided its fractionation using the elevated plus maze (EPM) in mice as a model of anxiety. A single treatment of AV extract markedly increased the percentage time spent on the open arms of the EPM in two distinct concentration ranges of 22.5–30 and 100–125 mg/kg p.o., respectively, indicating a putative anxiolytic-like activity. Fractions showing anxiolytic effects in concentrations equal to 30 or 125 mg/kg of whole extract were antagonized using the benzodiazepine antagonist flumazenil (3 mg/kg i.p.) or the 5-HT_{1A} receptor antagonist WAY-100635 (0.5 mg/kg i.p.). All active fractions in a concentration equal to 125 mg/kg were effectively blocked by the benzodiazepine antagonist flumazenil, while the anxiolytic activities of fractions in the lower dose equivalent to 30 mg/kg of whole extract were inhibited by the 5-HT_{1A} receptor antagonist WAY-100635. Through further separation of AV fractions it was possible to isolate and characterize the flavonol kaempferol which showed an anxiolytic-like activity in concentrations from 0.02 to 1.0 mg/kg p.o. The anxiolytic activity of kaempferol was partially antagonized by concomitant administration of flumazenil, but not by WAY-100635. In conclusion, our study clearly demonstrates that AV extract possesses anxiolytic-like activity and that at least one of its flavonoids, kaempferol, can elicit the same kind of neuropharmacological activity.

Keywords: Anxiolytic effect; Benzodiazepines; Elevated plus maze; *Apocynum venetum*; GABA receptor; Serotonin receptor; Kaempferol

Introduction

Anxiety disorders are among the most common mental disorders besides depressive disorders with approximately one-eighth of the world population affected at some point in their life (Eisenberg 1998). The total economic burden of this multi-symptom complex was estimated to be $42 billion for the United States alone in 2005 (Devane et al. 2005). Major drug classes for the treatment of anxiety disorders are benzodiazepines and selective serotonin-reuptake inhibitors (SSRIs). (Kunovac and Stahl 1995). All drug classes currently used are associated with side effects that vary in occurrence and severity. While the onset of action for the benzodiazepines is shortest, these drugs are associated with hepatotoxicity and cause insomnia and muscle relaxation which impair normal daily life (Lader and Morton 1991). SSRIs have a wider safety margin and are linked to less severe side effects, but the

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onset of action is usually long with 3–4 weeks (Sheehan and Sheehan 2007). These considerations implicate the search for new anxiolytic compounds that have a fast onset of action present with less side effects and a wider safety margin.

Apocynum venetum (AV, Apocynaceae) is a perennial shrub widely distributed throughout the Mediterranean area and northwestern China. Teas prepared from the flavonoid-rich leaves of AV have been used in traditional Chinese medicine as an important treatment for hypertension, nephritis, and neurasthenia (Yongxing and Shuying 1989). Recently, teas prepared from AV leaves have become a popular healthy beverage in Japan and are marketed as anti-aging nutritional supplements. The leaves seem to be free of alkaloids typical for the Apocynaceae family but they are rich in flavonoids (Li and Yuan 2006). A previous study evaluated the antidepressant activity of AV using the forced swimming test (FST) in rats and showed activities at 2 distinct concentrations of 30 and 125 mg/kg comparable to the antidepressant imipramine (Butterweck et al. 2001). In another study from our workgroup, the anxiolytic activity of AV was investigated using the elevated plus maze (EPM) animal behavior model in mice after acute treatment (Grundmann et al. 2007). In the present study, a more detailed dose–response profile for a hydroethanolic extract prepared from AV as well as the anxiolytic activities of fractions derived from the whole extract were examined using the EPM in mice. It was also the aim to isolate and identify those constituents possibly involved in this activity. In addition, it was of interest to further investigate the involvement of GABA and serotonin receptors in the anxiolytic-like effects of AV through co-administration of the benzodiazepine antagonist flumazenil and the 5-HT_{1A} receptor antagonist WAY 100635.

Materials and methods

Animals

Male BL6/C57J mice between 6 and 12 weeks old and with a mean weight of 27.3 ± 0.1 g were purchased from Harlan (Indianapolis, IN, USA). Mice were housed in cages of 5 at 20 ± 1 °C in a 12-h light/dark cycle. Tap water and standard food pellets were available ad libitum. Groups of 10–14 mice were randomly assigned to different treatment groups and tested in a varying order. Animals were tested repeatedly under the same experimental conditions. All experiments were carried out in a quiet room under controlled light conditions between 8:00 a.m. and 1:00 p.m. The sample size for each treatment group was determined by PS Power & Sample Size Calculation® (version 2.1.31, Plummer, D., Vanderbilt Medical Center, Nashville, TN, USA) for independent studies with 90% power, 95% confidence interval, expected standard deviation of 10, and expected difference of 15. The result was 10 mice per treatment group. All animals were housed and all experiments performed according to the policies and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida, Gainesville, USA (NIH Publication #85-23).

Drugs

Preparation of Apocynum extract and fractions: Leaves of AV (500 g) were refluxed for 1 h in aqueous ethanol (70% v/v, 300 ml) twice and the combined alcoholic extract was evaporated to dryness (140 g). The extract (67.5 g) was dissolved in hot water (1000 ml), and adjusted to pH 3.0 with sulfuric acid, then filtered. The filtrate was chromatographed on DIAION HP-20 (3.6 cm i.d. 318 cm) and eluted with water (1000 ml) and then aqueous ethanol (70% v/v, 1000 ml). The aqueous ethanol fraction was collected and evaporated to dryness to obtain AV extract (21 g) (DER 11-13:1). The extract was standardized to contain 3.5% hyperoside and 3.2% isoquercitrin, respectively.

Hyperoside and isoquercitrin were in-house purified (95% purity) by Tokiwa Phytochemical (Tokiwa Phytochemical Co. LTD, Chiba, Japan), chlorogenic acid was purchased from Acros (Acros distributed by Nagase & Co., Tokyo, Japan, 98% purity), quercetin was purchased from Sigma (Sigma-Aldrich K.K., Tokyo, Japan, 98% purity), kaempferol, catechin, epicatechin, and epigallocatechin were purchased from Funakoshi (Funakoshi Co. LTD, Tokyo, Japan, 95–99% purity) and gallocatechin was bought from Nagara (Nagara Science Co. LTD, Nagase, Japan, 95% purity).

Analytical conditions

The extract was dissolved in 50% (v/v) ethanol to a concentration of 1 mg/ml followed by filtration through a 0.45 μm nylon membrane filter and subjected to LC-DAD analysis. The analysis was performed on an Acquity UPLC-SQD system (Waters, Milford, MA) using a BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters) followed by a diode array detection at 254 nm and a mass spectrometer with electron spray ionization source. HPLC conditions were as follows: solvent A, H_2O/0.1% HCOOH; solvent B, CH_3CN/0.1% HCOOH; linear gradient, initial percentage of B (10%) to 10 min (20%) and to 15 min (50%); column temperature, 40 °C; flow rate, 0.2 ml/min. To detect target compounds specifically, selected ion recording (SIR) mode was used. MS parameters were as follows: ionization mode, positive; sheath gas, nitrogen; source