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Rhus parviflora and its biflavonoid constituent, rhusflavone, induce sleep through the positive allosteric modulation of $GABA_A$-benzodiazepine receptors

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ABSTRACT

Ethnopharmacological relevance: *Rhus parviflora* is referred as 'Tintidikah' in traditional medicinal system of south Asia (Ayurveda). It is used in treatment of *Vāta vikāra*, a condition related to neurological complications as well as cure for stomach disorders.
Materials and methods: Dried and powdered fruits of *R. parviflora* were extracted with 80% aqueous methanol (RPME). The concentrated extract was successively partitioned with distilled water (DW), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). All extracts, as well as isolated biflavonoids from *R. parviflora*, were evaluated for their affinity to the benzodiazepine binding site of $GABA_A$ receptor. The sedative-hypnotic effects of the fractions were evaluated by measuring sleep latency and sleep duration during pentobarbital-induced sleep in mice after oral administration of the extract fractions.
Results: Oral administration of RPME (125 mg/kg, 250 mg/kg, 500 mg/kg, and 1000 mg/kg) produced a dose-dependent decrease in sleep latency and an increase in sleep duration in mice treated with pentobarbital. The methanol extract produced a hypnotic effect that was fully blocked by ^{3}H-Ro 15-1788 flumazenil (FLU). Further, among the solvent fractions, the ethyl acetate fraction exhibited significant activity. Among the isolated compounds, biflavonoids mesuaferrone B (**1**), rhusflavone (**3**), and agathisflavone (**4**) competitively inhibited FLU binding with a K_i of 0.280 μM, 0.045 μM, and 0.091 μM, respectively. In addition, analysis of the sedative-hypnotic effects of rhusflavone, as well as those of the ethyl acetate, *n*-butanol, and distilled water fractions revealed that the modulation of both the ethyl acetate fraction and biflavonoid rhusflavone (**3**) are the most potent in inducing sleep.
Conclusion: The presence of conjugated ketone and C6-C8″ biflavonoid linkage in rhusflavone may be responsible for BZD-site of the $GABA_A$ leading to decrease in sleep latency and increase sleep duration.

1. Introduction

Nepalese sumac *Rhus parviflora* Roxb. (Anacardiaceae) is known as 'Tintidikah' in Sanskrit language, and is distributed in Nepal, northern India, Bhutan and Sri Lanka at the altitudinal range of 700–1100 m (Press et al., 2000). It is recorded in Ayurvedic pharmacopoeia as having therapeutic uses for *Vāta vikāra* (Anonymous, 2006), the complications related to neurological disorders including anxiety, insomnia, epilepsy, and rheumatoid arthritis. In Nepal fruits of *R. parviflora* are also used for human consumption (Bajracharya, 1980) and decoction of fruit or stem-bark is used for cure of dysentery (Bhattarai, 1991).

Insomnia is widespread malady, with 10–15% of the adult populations suffering from chronic insomnia and an additional 25–35% with transient or occasional insomnia (Doghramji, 2006). Sedative-hypnotic drugs, including benzodiazepines, non-benzodiazepines, gamma-aminobutyric acid ($GABA_A$) receptor agonists, antidepressants [serotonin $(5\text{-HT})_2$ receptor antagonist)] and antihistamines (Borja and Daniel, 2006), are available treatment options. However, the possibility of tolerance and dependence (Fang et al., 2010) is inevitable in long-term use. Therefore, there is need and opportunity to find natural products that enhance sleep quality without producing negative side-effects. Svenningsen et al. (2006)

Abbreviations: BBB, blood-brain barrier, BZD, benzodiazepine; c.c., column chromatography; $CHCl_3$, chloroform; CMC, carboxymethyl cellulose; CNS, Central nervous system; CON, control group; DPM, disintegrations per minute; DW, distilled water; DZP, diazepam; EtOAc, ethyl acetate; FAB-MS, fast atom bombardment mass spectrometry; FLU, flumazenil; $GABA_A$, gamma-aminobutyric acid; IC_{50}, Half-maximal inhibitory concentration; ICR, imprinting control region; i.p., intraperitoneal injection; IR, infra-red, MeOH, methanol; *n*-BuOH, *n*-butanol; NMR, nuclear magnetic resonance spectroscopy; *R. parviflora*, *Rhus parviflora* Roxb.; RPME, *Rhus parviflora* methanol extract; SD, Sprague Dawley; SEM, standard error of the mean; TLC, thin layer chromatography, V_e/V_t, elution volume/total volume

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reported that the biflavones agathisflavone and amentoflavone from *R. pyroides* exhibit good affinity for $GABA_A$/benzodiazepine receptors. Furthermore, low catecholamine may be causative factor of epilepsy and attention deficient-disorder, Kaufmann et al. (2009). Sapkota et al. (2009) reported regulation of catecholamine biosynthesis by *Rhus verniciflua* extract. Therefore, species belonging to genus *Rhus* may have promising neurotransmitter compounds having therapeutic usage for insomnia and epilepsy.

In our continuing search for active materials from natural sources, the methanolic extract of *R. parviflora* fruit was found to exhibit significant $GABA_A$-BZD receptor binding capacity. Next, the concentrated extract was suspended in water and partitioned successively with EtOAc and *n*-BuOH. Since the EtOAc fraction showed the most efficient binding affinity, it was subjected to repeated silica gel, ODS, and Sephadex LH-20 column chromatography, leading to the isolation of five biflavonoids, namely mesuaferrone B (**1**), rhusflavanone (**2**), rhusflavone (**3**), agathisflavone (**4**), and cupressuflavone (**5**) for the first time from *R. parviflora*. The compounds were identified by interpretation of spectroscopic data including NMR, MS, and IR. The $GABA_A$ binding affinities of biflavonoids were investigated and the hypnotic potentialities of the EtOAc, *n*-BuOH and DW fractions were evaluated using the pentobarbital-induced sleep test in ICR mice to find compounds responsible for inducing sleep through the positive allosteric modulation of $GABA_A$-benzodiazepine receptors.

2. Materials and methods

2.1. Plant material

Fruits of *R. parviflora* were collected from Salyan (28° 22′ 31″ N, 82° 9′ 42″ E), Nepal in February 2010, and were identified by Prof. Damodar Prasad Joshi, Central Department of Environmental Science, Tribhuvan University, Nepal. The voucher specimen (KHU100309) is deposited in the Natural Products Chemistry Laboratory, Kyung Hee University, Yongin, Korea.

2.2. Phytochemical constituents

The fruits of *R. parviflora* contain echinulin, trimethyl citrate and citric acid 2-methyl ester (Talapatra et al., 1993, 2001). Recent investigation of Shrestha (2011) determined presence of a flavanone naringenin as major component together with flavonoids, biflavonoids, flavonoid glucosides, triterpenoid, sterols, lipids, lignan, lignan glycosides, sterols, phenolic acid and phenolic acid glycosides from RPME.

2.3. Animals

To obtain a membrane preparation for the $GABA_A$-BZD receptor-binding assay, 200–250 g male SD (Sprague-Dawley) rats were used. In the pentobarbital-induced sleep test, male ICR (Imprinting Control Region) mice weighing 18–22 g were used. All animals were purchased from Koatech Animal Inc. (Pyeongtaek, Korea) and were housed with food and water *ad libitum* at 24 °C at a controlled humidity of 55% in a room maintained on a 12 h light/dark cycle (light on at 9:00 AM). All procedures involving animals were conducted in accordance with the animal care and use guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission number: KFRI-M-09118).

2.4. Dose selection

RPME was tested on range of dose levels 125 mg/kg, 250 mg/kg, 500 mg/kg, and 1000 mg/kg. The human equivalent dose (HED) of 20 mg/kg in mice is 1.62 mg/kg calculated on the basis of body surface area, or it is equivalent to 113 mg for 70 kg person (Liu et al., 2006). Further test to establish the exact lethal dose was not done as we used 2 mg/kg of diazepam (DZP) as a positive control and there was no sign of toxicity symptoms even at dose up to 3000 mg/kg for RPME in ICR mice.

2.5. Drugs, chemicals, and reagents

The organic solvents for solid extraction and solvent fractionation were 1st grade reagents supplied by Daejung Chemical Ltd. (Seoul, Korea). SiO_2 and ODS resins used for column chromatography were Kiesel gel 60 (Merck, Darmstadt, Germany), and LiChroprep RP-18 (Merck), respectively. TLC analysis was carried out using Kiesel gel $60F_{254}$ and RP-18, $F_{254}S$ (Merck) was detected using UV lamp Spectroline Model ENF-240 C/F (Spectronics Corporation, NY), and 10% H_2SO_4 solution. Deuterium solvents were purchased from Merck Co. Ltd and Sigma-Aldrich Inc. (St. Louis, MO, USA).

Pentobarbital was purchased from Hanlim Pharm. Co. Ltd. (Seoul, Korea). A $GABA_A$-BZD agonist DZP (Myungin Pharm. Co. Ltd., Seoul, Korea), was used as a reference sedative-hypnotic drug. FLU and picrotoxin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). For the $GABA_A$-BZD receptor-binding assay, the radioligand FLU (Ro 15-1788; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used. All other chemicals and reagents used were of the highest grade available.

2.6. Instrumentation

Melting points were determined on a Fisher–John's apparatus (Fisher Scientific, Chicago, USA). Optical rotations were measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan). IR spectrum was obtained from a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). FAB-MS was recorded on a JEOL JMSAX-700 (Tokyo, Japan). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR spectrometer (Palo Alto, CA).

2.7. Preparation of *R. parviflora* methanol extract and solvent fractions

Dried and powdered fruits (6 kg) of *R. parviflora* were extracted at room temperature with 80% MeOH (25 L × 3) for 24 h, which resulted in a yellowish brown concentrated extract (1440 g). The MeOH extract was suspended in H_2O (6 L) and successively partitioned with EtOAc (6 L × 3), and *n*-BuOH (6 L × 3), yielding concentrated extract in EtOAc (RPE, 48 g), *n*-BuOH (RPB, 173 g), and distilled H_2O (DW, 1219 g) fractions.

2.8. Isolation of biflavonoid constituents from the *R. parviflora* ethyl acetate fraction

The concentrated *R. parviflora* EtOAc fraction (RPE, 48 g) was subjected to silica gel column chromatography (c.c.) (ϕ 14 × 12 cm) and eluted with *n*-hexane-EtOAc [(10:1 → 3:1 → 1:1, 20 L of each), and $CHCl_3$-MeOH (6:1 → 1:1, 10 L each), resulting in 22 fractions (RPE-1 to RPE-22). Fraction RPE-13 (2.41 g, V_e/V_t 0.59–0.80) was subjected to Sephadex LH-20 c.c. (ϕ 2.5 × 45 cm), and was eluted with MeOH-H_2O (2:1, 500 mL, → 2:1, 1.5 L) to yield 18 subfractions (RPE-13-1 to RPE-13–18), and isolation of compound **1** at RPE-13–17 [30 mg, V_e/V_t 0.59-0.80, TLC (RP-$18F_{254}$S) R_f 0.55, in MeOH-H_2O (3:1)]. Fraction RPE-13-13 (474.8 mg, V_e/V_t 0.16-0.33) was subjected to ODS c.c. (ϕ 3 × 4 cm), and was eluted with MeOH-H_2O (1:1.5, 500 mL → 2:1, 1.5 L) resulting into nine subfractions (RPE-13-13-1 to RPE-13-13-9) and