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6-Methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS

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Abstract

Valerian is an ancient tranquillizing drug obtained from the underground organs of several *Valeriana* species. Its active principles were assumed to be terpenoids in the form of valepotriates and/or as components of the essential oil. However, unknown active compounds were not discarded and synergic effects were suspected.

We have recently isolated 6-methylapigenin (MA) from *Valeriana wallichii* and proved that it is a benzodiazepine binding site (BDZ-bs) ligand [Planta Med. 68 (2002) 934].

The present paper is the first report of the presence of 2*S*(−)-hesperidin in valeriana and describes that it has sedative and sleep-enhancing properties. MA, in turn, was found to have anxiolytic properties and was able to potentiate the sleep-enhancing properties of hesperidin (HN).

MA and HN are new members of the growing family of natural flavonoids with activity on the CNS, and their properties suggest that they are promising drug leads in the field.

Keywords: Valeriana; Flavonoids; 6-Methylapigenin; Hesperidin; CNS; Benzodiazepine binding site; $GABA_A$ receptor; Sedation; Hypnosis; Anxiolysis

1. Introduction

Several *Valeriana* species are the source of a natural drug known since 800 BC and is now being used as a sedative with such popular success that it has even been called "the Valium of the 19th century" (Hobbs, 1989).

Multiple substances in the valeriana extracts are held responsible for their effects, and most important among them are the terpenoid esters named valepotriates, their decomposition products, the baldrinals and various components of the essential oil, in particular, the valerenic acid derivatives (Houghton, 1999). However, some experimental facts cast doubts upon the real involvement of these substances, namely, the variable compositions of different valerianas with similar activities (Hobbs, 1989; Houghton, 1999), their failure to modify the glucose metabolism in brain, a known test to detect activity in the CNS (Hölzl, 1997), and finally, their frequent absence in many pharmaceutical formulations (Bos et al., 1996). Hence, the existence of yet unknown active substances in valeriana remains a valid alternative that is supported by the recent isolation from *Valeriana wallichii* DC and detection in *V. officinalis* L., of 6-methylapigenin (MA), a flavone derivative that is a ligand for the benzodiazepine binding site (BDZ-bs) of the $GABA_A$ receptor (Wasowski et al., 2002). This finding is here reinforced by the isolation and identification in both of these *Valeriana* species, of 2*S*(−)-7-rhamnoglucosyl-hesperetin or hesperidin (HN), a flavanone glycoside with sedative and sleep-enhancing properties. This paper is the first report of the presence of HN in valeriana and describes the biochemical and pharmacological properties of both flavonoids, MA and HN. We estimate that their joint in vivo effects explain

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the major properties of *V. wallichii*, and probably of *V. officinalis*.

2. Methods

2.1. Plant material

V. wallichii DC and *V. officinalis* L. (Valerianaceae) were obtained from a local commercial source. Their identification was done at the Botany Museum of the School of Pharmacy in Buenos Aires, where the voucher specimens J.L.A. 10288 and 10392 BAF, respectively, were deposited.

2.2. Subjects

Adult male Wistar rats weighing 250 g were used for biochemical experiments. Adult male Swiss mice weighing 25–30 g were used for pharmacological assays. Animals were housed in a controlled environment, with free access to food and water and maintained on a 12:12-h day/night cycle.

Housing, handling, and experimental procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

2.3. Biochemical experiments

The binding of ^{3}H-flunitrazepam (^{3}H-FNZ) to BDZ-bs (81.8 Ci/mmol; New England Nuclear, NEN) in washed crude synaptosomal membranes from rat cerebral cortex was carried out as described by Medina et al. (1990). For each assay of the inhibition experiments, triplicate samples of the membranes containing 0.2–0.4 mg of protein were suspended in a final volume of 1 ml of 25 mM Tris–HCl buffer, pH 7.3, in the presence of a solution of the sample assayed. The incubation was carried out at 4 °C for 60 min with 0.6 nM ^{3}H-FNZ. Nonspecific binding was determined in parallel incubations in the presence of 10 μM FNZ, and was represented 5–15% of the total. The assays were terminated by filtration under vacuum through Whatman GF/A glass-fiber filters and three washes with 3 ml each of incubation medium. Filters were counted after addition of Optiphase "Hisafe" 3 (Wallac, Turku, Finland) liquid scintillation cocktail.

Additional binding studies were performed as described elsewhere: ^{3}H-8-hydroxydipropylaminetretraline (^{3}H-8-OH-DPAT, NEN) for 5-hydroxytriptamine-type 1A receptors (5-HT$_{1A}$) (Nénonéné et al., 1994); ketanserine hydrochloride [ethylene-^{3}H] (^{3}H-ketanserine, NEN) for 5-HT$_2$ receptors (Reyes-Parada et al., 1996); ^{3}H-amine-3-hydroxy-5-methyl-4-isoxazolepropionic acid (^{3}H-AMPA, NEN) for AMPA glutamatergic receptors (Cammarota et al., 1997); and chloro-*N*(6)-cyclopentyladenosine, 2-[cyclopentyl-2,3,4,5-^{3}H] (^{3}H-CCPA, NEN) for adenosine$_1$ receptors (El-Ani et al., 1997), with slight modifications.

2.4. Behavioral experiments

2.4.1. Elevated plus-maze test

Anxiolysis was measured in mice by the increase in the number of entries in the open arms and/or the time spent in the open arms in an elevated maze formed by two open and two closed intersecting arms (Pellow et al., 1985). The test was performed in the same session immediately after the locomotor activity assay as described by Viola et al. (1994) and Wolfman et al. (1994). It is widely validated for rodents (Lister, 1987; Pellow et al., 1985) and possesses several advantages over other tests for measuring anxiety (Dawson and Tricklebank, 1995).

2.4.2. Locomotor activity assay

Locomotor activity was measured in a box by optical means (Opto-Varimex apparatus) according to Viola et al. (1994). An increase in the number of transitions through the light beams reflects augmented locomotor activity.

2.4.3. Holeboard test

Sedation was evidenced by the decrease in exploratory behavior of mice in an open field with holes in the floor (File and Pellow, 1985). The test was performed according to Viola et al. (1994) and Wolfman et al. (1994). The number of head dips and the time spent head-dipping as well as the rearings were counted during 5 min. A decrease in these parameters reveals a sedative behavior.

2.4.4. Sodium thiopental-induced sleeping-time assay

Sodium thiopental (35 mg/kg) was intraperitoneally injected 20 min after the vehicle or the drug. The disappearance and the reappearance of the righting reflex were considered indications of duration of sleep (Anca et al., 1992).

2.4.5. Horizontal wire test

This test was carried out as previously described (Viola et al., 1994, 1995; Wolfman et al., 1994). The test takes place after two trials, performed at 5-min intervals. A myorelaxant drug impairs mice to grasp the wire (Bonetti et al., 1982).

2.5. Drugs solutions and injection procedures

The dried fractions obtained from *V. wallichii*, MA and HN isolated from *V. wallichii*, HD (Sigma) and diazepam (Hoffmann-La Roche), were dissolved by the sequential addition of 10% dimethylsulfoxide, 10% ethanol, and 80% saline. Sodium thiopental (Abbott) was dissolved in distilled water. The rodents were intraperitoneally injected 20 min before the tests, except when indicated. The volume of intraperitoneal injections was 0.15 ml/30 g of body weight. The potentiating effects were tested performing coadministrations of MA plus HN. In each session, control mice were

tested in parallel with those animals receiving drug treatment.

2.6. Statistical analyses

ANOVA was used when several mice treatments were compared. Post hoc comparisons between individual treatments and controls were made using Dunnett's multiple comparison test. Nonparametric Dunn's multiple comparison test was used when sleeping times were compared.

2.7. Isolation and identification of MA and HN

Dry *V. wallichii* roots and rhizomes were submitted to the extraction and fractionation scheme shown in Fig. 1.

Fig. 2 shows the results of analytical high-performance liquid chromatographies (HPLCs) of the ethyl ether extract and of the aqueous remaining phase described in Fig. 1.

HPLC fractionations were performed using an LKB Pharmacia apparatus for analytical HPLCs and an ISCO apparatus, adapted for high liquid fluxes, to perform semi-preparative or preparative HPLCs. C-18 reversed phase Vydac columns (The Separation Group, Hesperia, CA, USA) were used for preparative (5 μm, 2.2 × 25 cm), semi-preparative (5 μm, 1 × 25 cm), and analytical (5 μm, 0.46 × 25 cm) purposes. Each extract was properly injected into the column and eluted using an aqueous acetonitrile (ACN) gradient, as indicated in Fig. 2. Monitoring of the effluent was at 280 nm.

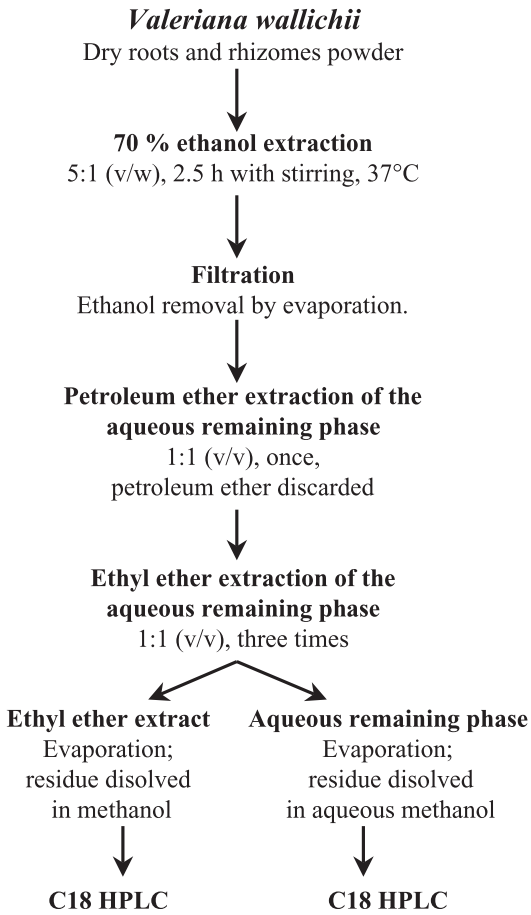
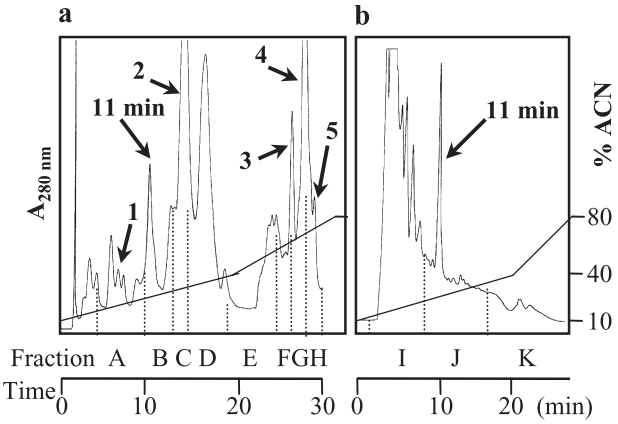


Fig. 1. Flow sheet of the *V. wallichii* fractionation scheme.



Receptors and activity	Fractions / binding A	B	C	D	E	F	G	H	I	J	K
BDZ-bs	-	-	-	+++	-	-	-	-	-	-	-
5 HT_{1A}	+	-	+	+	+	+	-	+	-	-	-
5 HT_2	-	-	-	-	-	-	-	-	-	-	-
AMPA-glutamatergic	-	-	-	-	-	-	-	-	-	-	-
Adenosine$_1$	-	-	-	+	-	-	-	-	-	-	-
Sedation-Hypnosis				++						+++	

Fig. 2. (a) and (b) are representative analytical HPLC chromatograms of *V. wallichii* extracts: (a) Ethyl ether extract (see Fig. 1); (b) Aqueous remaining phase (see Fig. 1). HPLC fractionations were performed using an LKB Pharmacia apparatus for analytical HPLC using a C-18 reversed-phase Vydac column. Each extract was properly injected into the column and eluted using an aqueous ACN gradient, as indicated in the figure, at a flow rate of 1 ml/min. The detection was done at λ = 280 nm. The numbered arrows in the HPLC diagrams indicate the retention times of the following valeriana components: 1 and 2: baldrinals; 3: acevaltrate; 4: didrovaltrate; and 5: valtrate. In (a) and (b), 11 min indicate the eluted peak with the retention time of the novel compound HN described in this work. Valerenic acid, which is absent in *V. wallichii*, but present in *V. officinalis*, has a retention time intermediate between those of compounds 4 and 5. For biochemical and pharmacological testing fractions, A to K were recovered from preparative and semi-preparative HPLCs fractionations performed under similar conditions as described for the analytical fractionations. The capacity of the different fractions obtained to bind to several brain receptors related to anxiolysis, as well as the sedative and hypnotic properties of the fractionated extracts, measured as indicated in the Methods section, is also shown. The binding assays were performed with an amount of each fraction equivalent to that obtained from 0.1 g dry *V. wallichii* and were indicated as inhibition ≥ 60–80%, (+++); inhibition ≥ 10–20%, (+); and inhibition < 10%, (−). The in vivo assays were performed in mice intraperitoneally injected with an amount of the extracts equivalent to that obtained from 1 g of dry *V. wallichii* powder, 20 min before the test.

Fractions A to K (Fig. 2) were recovered for use in biochemical and pharmacological testings. They were submitted to in vitro binding assays to the following brain receptors, all related to anxiolytic effects: BDZ-bs, 5-HT_{1A}, 5-HT_2, AMPA glutamatergic and adenosine$_1$.

The results of the binding assays shown in Fig. 2 indicated that high-affinity ligands for the BDZ-bs were present in the single discrete fraction D not containing known compounds. There was, however, some proportion of ligands in D, also recognizing 5-HT_{1A} and adenosine receptors. The low binding activity for the receptor 5-HT_{1A} found in fractions A, C, E, F, and H was probably related to the known components of valeriana present in them and was not further investigated.

The unfractionated ethyl ether extract and the whole aqueous remaining phase were found to have sedative and hypnotic effects in vivo, as indicated in Fig. 2.

The isolation of the BDZ-bs ligand from the ethyl ether extract of *V. wallichii* (and *V. officinalis*) as well as its identification as MA (Fig. 3) has already been published (Wasowski et al., 2002).

The isolation and purification of the active component in the aqueous remaining phase of Fig. 2 was bioguided using the sleeping-time assay. This compound was extractable from the aqueous phase with amyl alcohol, the extraction being performed three times with equal volumes of both phases. The solid residue from this extract, corresponding to 100 g of starting valeriana powder, was chromatographed on a silica gel column (4.5 × 20 cm, silica gel H, Sigma). The column was eluted with 20% methanol in chloroform. The active fractions were pooled. After partial evaporation of the solvent, the concentrate deposited a whitish precipitate that was collected by filtration and purified by recrystallization from ethanol. The UV, NMR, and mass spectral data permitted the identification of this compound as 2*S*(−)-7-rhamnoglucosyl-hesperetin, or HN (Fig. 3).

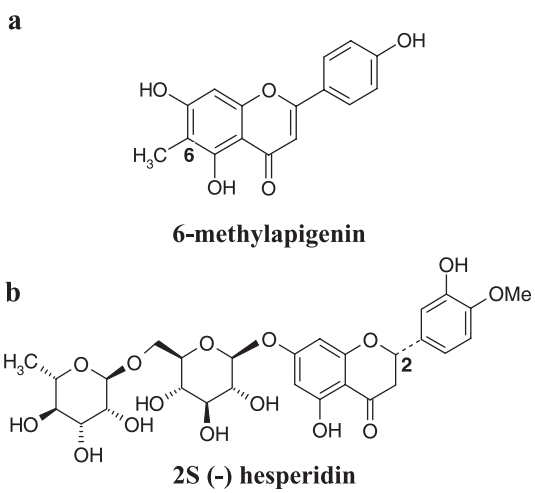


Fig. 3. (a) Molecular structure of 6-methyl-4′,5,7-tryhydroxyflavone or MA. (b) Molecular structure of 4H-1-benzopyran-4-one, 7-[[6-*O*-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-, (*S*)- or 2*S*(−)-hesperidin.

Spectroscopic measurements were done as follows: NMR on a 300-MHz Bruker apparatus with the sample dissolved in DMSO-d_6; UV–Vis in a Shimadzu 160A spectrophotometer with methanolic solutions; EIMS on a Shimadzu Mass-Spectrometer QP-5000 at 70 eV with direct probe inlet.

All the spectra of valeriana HN and authentic HN (Sigma) were identical: ^{1}H NMR δ_H 11.99 (1H, s, OH-5), 9.04 (1H, s, OH-3′), 6.90 (3H, m, H-2′, H-5′ and H-6′), 6.11 (2H, d, J=6 Hz, H-8 and H-6), 5.50 (1H, dd, J=3 and 12.3 Hz, H-2), 4.96 (1H, d, J=7 Hz, H-1 glucose), 4.40 (1H, d, J=6 Hz, H rhamnose), 3.76 (3H, s, CH_3-4′), 3.10–3.28 (m, OH-rhamnoglucosyl and H-3 trans), 2.80 (1H, d, J=3 Hz, H-3 cis), 1.06 (^{3}H, s, CH_3-rhamnose). ^{13}C NMR δ_C 197.1 (C-4), 165.1 (C-7), 163.0 (C-5), 162.5 (C-9), 147.9 (C-3′), 146.4 (C-4′), 130.9 (C-1′), 128.5 (C-6′), 118.0 (C-5′), 114.1 (C-2′), 112.0 (C-10), 103.3 (C-1 glucose), 99.4 (C-1 rhamnose), 95.5 (C-8), 78.4–68.3 (C-2, C-3, C-4 and C-5 glucose and rhamnose), 121.3 (C-1′), 66.0 (C-6 glucose), 55.7 (OCH_3-4), 17.9 (C-6 rhamnose). UV λ_{max} 331.5, 283.5, 210 nm.

To further characterize the aglycone moiety of the isolated compound samples of valeriana HN or authentic HN (Sigma) was hydrolyzed with boiling 1 M HCl for 1 h. The aglycones were extracted from the corresponding hydrolysates with ethyl ether. Each ether extract residues were submitted to spectroscopic analysis. All the spectra of valeriana HN and authentic HN aglycones, that is, hesperetin, were identical: ^{1}H NMR δ_H 12.11 (1H, s, OH-5), 10.75 (1H, s, OH-7), 9.05 (1H, s, OH-3′), 6.90 (3H, m, H-2′, H-5′ and H-6′), 5.87 (2H, dd, J=2 and 4.7 Hz, H-8 and H-6), 5.42 (1H, dd, J=3 and 12.3 Hz, H-2), 3.76 (3H, s, CH_3-4′), 3.17 (1H, m, J=4.7 and 12.3 Hz, H-3 trans), 2.73 (1H, d, J=3 Hz, H-3 cis). EIMS *m/z* 302 (M^+), 285, 259, 179, 150.

Samples from the hydrolysated aqueous phases remaining after the ethyl ether extraction were submitted to thin-layer chromatographies on silica gel on polyester sheets, with 254 nm fluorescent indicator (Sigma), developed with buthanol/acetic acid/ethyl ether/water (9:6:3:1 v/v) as the solvent and stained using a general reactive for sugars (aniline/diphenylamine/acetone/phosphoric acid). These chromatographies were performed alongside with authentic sugar standards. In both cases, the only sugars detected were glucose and rhamnose.

2.7.1. Optical activity of HN

The disaccharide rhamnose–glucose, or rutinose, is glycosidically attached to C-7 in hesperetin to give rise to HN (see Fig. 3). Rutinose has optical activity due to several asymmetric carbons, and its contribution to the optical activity of HN remains unchanged when the glycoside is submitted to mild treatments with alkali or acid. However, the bond between the asymmetric C-2 and the pyrone O is split by mild alkali giving rise to a chalcone, where the C-2 asymmetry is lost (Higby, 1943).

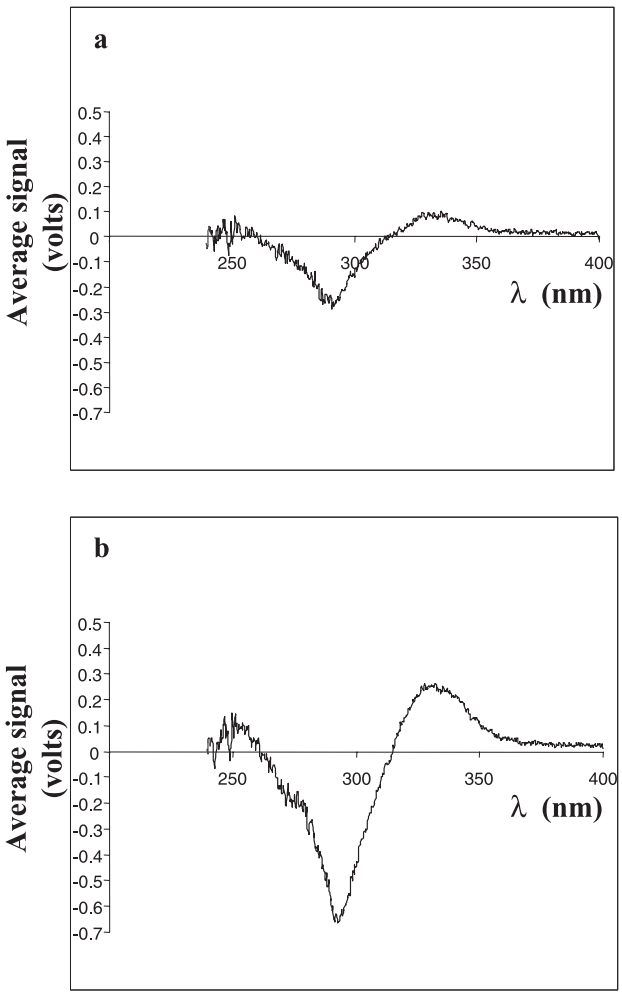


Fig. 4. Circular dichroism spectra of (a) recrystallized citrus HN (Sigma) and (b) valeriana HN. The spectra were recorded in a Jasco-20 spectropolarimeter, between 250 and 400 nm, with the samples dissolved in DMSO ($c = 0.4$ mg/ml). Each spectrum in this figure is the average of three measurements. The average signal, in volts, is an ellipticity equivalent, measured directly from the apparatus.

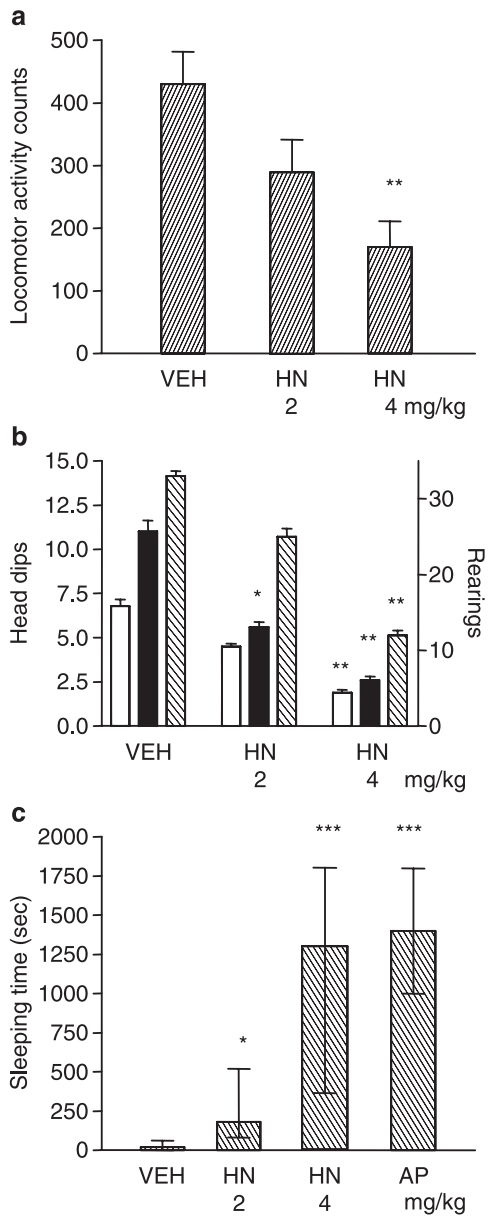


Fig. 5. Effects of HN on sedative and hypnotic behavior. (a) Mean ± S.E.M. of the ambulatory locomotor activity counts during a 5-min test session in an Opto-Varimex apparatus 20 min after an intraperitoneal injection of vehicle (VEH) or hesperidin (HN, 2 and 4 mg/kg). $^{**}P < .01$, significantly different from vehicle, Dunnett's multiple comparison test after ANOVA. Number of animals per group ranged between 10 and 11. (b) Mean ± S.E.M. of number of head dips (open bars, left scale) or time spent head-dipping in seconds (closed bars, left scale) and number of rearings (hatched bars, right scale) registered in a 5-min session in the holeboard test performed immediately after the measure of locomotor activity explained in (a). $^{*}P < .05$, $^{**}P < .01$, significantly different from vehicle, Dunnett's multiple comparison test after ANOVA. Number of animals per group ranged between 10 and 11. (c) Median (interquartile range) of sleeping time of mice in a sodium thiopental-induced sleeping time test 20 min after an intraperitoneal injection of vehicle, hesperidin (HN 2 and 4 mg/kg) or the aqueous phase (AP) obtained from 1 g of *V. wallichii* (150 mg of dry residue/kg). $^{*}P < .05$, $^{***}P < .001$, significantly different from vehicle, Dunn's multiple comparison test after Kruskal–Wallis nonparametric ANOVA. Number of animals per group ranged between 12 and 20.

The reversal of this reaction by acidification originates a racemic form of HN at C-2. This racemate however is optically active due to the presence of the rutinose moiety in the molecule.

The Index Merck records for the specific optical rotatory power of 2*S*-hesperidin: $[\alpha]_D^{20} = -76°$ ($c = 2$ in pyridine).

Our own results with HN obtained from *V. wallichii* and with authentic HN (Sigma), recrystallized from acetic acid, are HN (*V. wallichii*): $[\alpha]_D^{20} = -84.5°$ ($c = 0.02$ in pyridine) and HN (Sigma): $[\alpha]_D^{20} = -64.3°$ ($c = 0.02$ in pyridine).

The low rotatory power of the authentic HN (Sigma) can be explained by its extraction from citrus peels using alkaline solutions, later acidified, which brings about its partial racemization. We assume that the valeriana HN obtained by us is the pure 2*S*(−) natural form. The difference in absolute value of [α] with that in the Index Merck is probably due to the different concentrations used.

2.7.2. Circular dichroism (CD) studies

It is clear from the previous considerations that optical rotatory measurements are not suitable to ascertain the proportions of isomers in a particular sample of HN.

CD measurements, in contrast, are appropriate for this purpose since they detect the contribution of each absorption band of the molecule, and the sugar moiety, rutinose, does not interfere being devoid of absorptive bands in the UV.

Fig. 4 shows the CD spectra of recrystallized authentic HN (Sigma) and of valeriana HN, isolated in the present work. The spectra were recorded in a Jasco-20 spectropolarimeter, between 250 and 400 nm, which includes the typical UV bands of HN at 286, 289, and 330 nm.

Using the ellipticity recorded at 330 nm for both HNs, it can be calculated that the citrus HN is a mixture of 25% 2*S*(−) and 75% racemic forms. Hence, citrus HN has only approximately 63% of pharmacologically available S active form.

3. Results

3.1. Biochemical experiments

HN and hesperetin, at concentrations up to 100 μM, had no potency in displacing ^{3}H-FNZ binding to synaptosomal membranes of rat cerebral cortex. HN (10 μM) did not displace ^{3}H-AMPA, ^{3}H-8-OH-DPAT, ^{3}H-ketanserine, and ^{3}H-CCPA bindings to AMPA glutamatergic, 5-HT_{1A}, 5-HT_2 and adenosine$_1$ receptors, respectively.

3.2. Sedative and sleep-enhancing effects of HN

The depressant action of HN was determined in three different behavioral tests. A dose of 4 mg/kg of HN decreased the ambulatory locomotor activity ($P<.01$, Fig. 5a), reduced the exploration of holes and the number of rearings performed in the holeboard test ($P<.001$, Fig. 5b), and increased the sodium thiopental-induced sleeping time ($P<.001$, Fig. 5c). The effect of this dose of HN on sleep was comparable to that found after the administration of the aqueous phase dry residue obtained from 1 g of *V. wallichii* ($P<.001$, Fig. 5c).

The depressant effect of HN was dose-dependent. The intraperitoneal injection of 2 mg/kg of HN had a moderate action on the time spent head-dipping ($P<.05$, Fig. 5b) and on thiopental-induced sleeping time ($P<.05$, Fig. 5c).

HN isolated from citrus (Sigma) at doses of 4 and 9 mg/kg showed a milder sleep-enhancing effect compared to the valerian HN (Table 1). Citrus HN at a dose of 4 mg/kg had no significant sedative effect in the holeboard test (data not shown). Moreover, neither valerian nor citrus HN at a dose of 4 mg/kg produced a myorelaxant effect as detected in the wire test (data not shown).

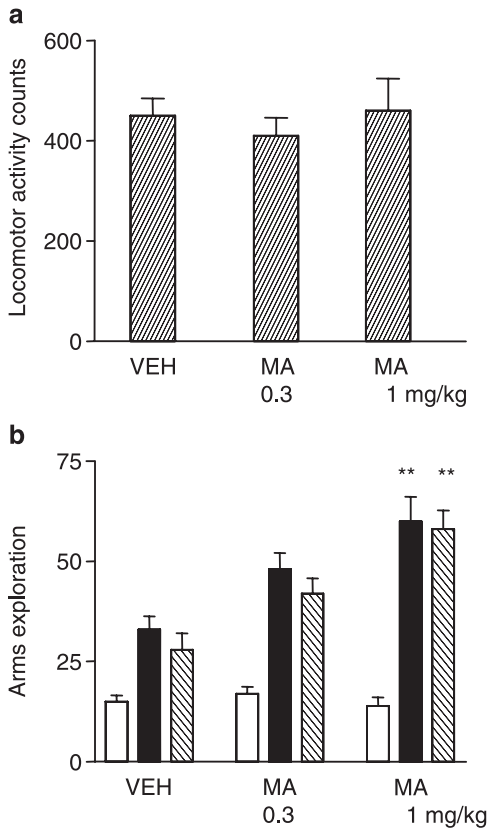


Fig. 6. Effect of MA on anxiolytic behavior. (a) Mean ± S.E.M. of the ambulatory locomotor activity counts during a 5-min test session in an Opto-Varimex apparatus 20 min after an intraperitoneal injection of vehicle (VEH) or MA (0.3 and 1 mg/kg). $P>.05$, ANOVA. Number of animals per group ranged between 12 and 14. (b) Mean ± S.E.M. of number of total arm entries (open bars), percentage of number of open arm entries (closed bars), and percentage of time in open arms (hatched bars) registered in a 5-min session in the elevated plus maze performed immediately after the measure of locomotor activity explained in (a). ** $P<.01$, significantly different from vehicle, Dunnett's multiple comparison test after ANOVA. Number of animals per group ranged between 12 and 14.

Table 1
Effectiveness of valeriana and citrus (Sigma) HNs on sleeping time of mice

Sample	Dose (mg/kg)	*n*	Sleeping time median (interquartile range) (s)
VEH	–	21	0 (0–21)
Valeriana HN	2	15	197 (83–522)*
	4	14	1290 (565–1605)**
	9	6	1800 (1638–1800)**
Citrus HN	4	7	660 (165–978)*
	9	6	1440 (1050–1710)**

Median (interquartile range) of sleeping time of mice given a sodium thiopental intraperitoneal dose (35 mg/kg) 20 min after an intraperitoneal injection of vehicle (VEH) or different doses of HN. The sleeping time was measured as the time spent between disappearance and reappearance of righting reflex (see Methods). *n* = number of mice.

* $P<.05$, different from vehicle, Dunn's comparison test after Kruskal–Wallis nonparametric ANOVA test.

** $P<.001$, different from vehicle, Dunn's comparison test after Kruskal–Wallis nonparametric ANOVA test.

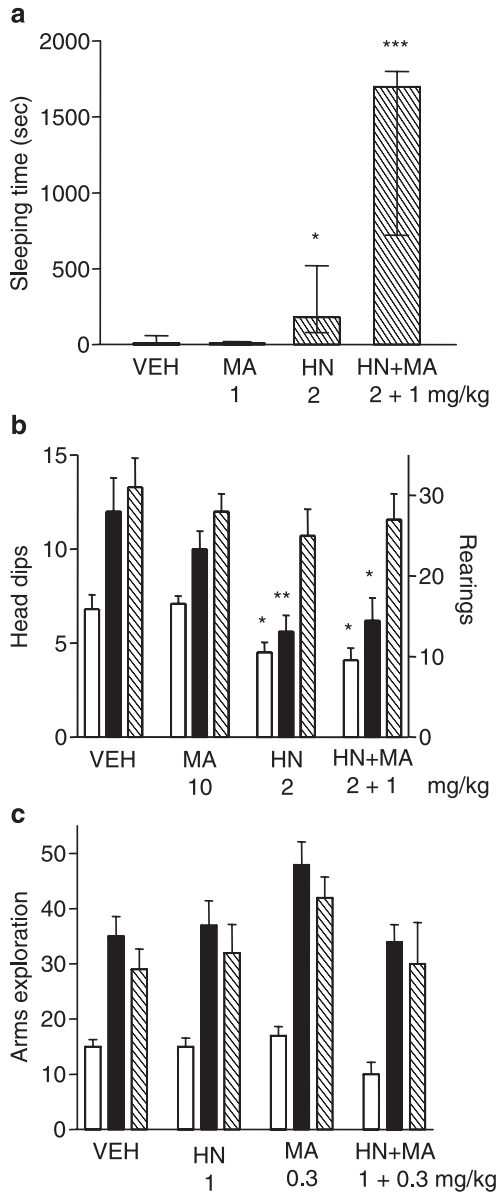


Fig. 7. Potentiation of HN sleep-enhancing action by 6-methylapigenin and no-potentiation of sedative or anxiolytic properties. (a) Median (interquartile range) of sleeping time of mice given a sodium thiopental-induced sleeping time test 20 min after an intraperitoneal injection of vehicle (VEH), MA (1 mg/kg), HN (2 mg/kg), or both drugs coinjected (HN 2 mg/kg + MA 1 mg/kg). $*P<.05$, $***P<.001$, significantly different from vehicle, Dunn's multiple comparison test after Kruskal–Wallis nonparametric ANOVA. Number of animals per group ranged between 10 and 15. (b) Mean ± S.E.M. of number of head dips (open bars) or time spent head-dipping in seconds (closed bars) and number of rearings (hatched bars) registered in a 5-min session in the holeboard test performed 20 min after the intraperitoneal injection of vehicle (VEH), MA (10 mg/kg), HN (2 mg/kg) or both drugs coinjected (HN 2 mg/kg + MA 1 mg/kg). $*P<.05$, $**P<.01$, significantly different from vehicle, Dunnett's multiple comparison test after ANOVA. Number of animals per group ranged between 8 and 10. (c) Mean ± S.E.M. of number of total arm entries (open bars), percentage of number of open arm entries (closed bars), and percentage of time in open arms (hatched bars) registered in a 5-min session in the elevated plus maze performed 20 min after the intraperitoneal injection of HN (1 mg/kg), MA (0.3 mg/kg), or both drugs coinjected (HN 1 mg/kg + MA 0.3 mg/kg). $P>.05$, ANOVA. Number of animals per group ranged between 10 and 16.

3.3. Anxiolytic-like properties of 6-methyl apigenin

MA at dose of 1 mg/kg produced anxiolytic-like effects as determined by the increase in the percentage of open arm entries and the increase in time spent in the open arms of the plus maze ($P<.01$, Fig. 6b). No changes were observed in the total arm entries ($P>.05$, Fig. 6b) or in the ambulatory locomotor activity ($P>.05$, Fig. 6a) between experimental groups, confirming the selective increment in open arms exploration. Moreover, in Fig. 7b, it is shown that a 10-fold higher dose of MA (10 mg/kg) was devoid of sedative action evaluated in the holeboard test.

3.4. Potentiation of HN sleep-enhancing action by 6-methyl apigenin

As shown in Figs. 5c and 7a, the intraperitoneal administration of HN at a dose of 2 mg/kg increased the sleeping time induced by sodium thiopental ($P<.05$). This HN hypnotic action was potentiated by the addition of MA at the anxiolytic dose of 1 mg/kg ($P<.001$, Fig. 7a). This action was not attributable to an additive effect because the administration of 1 mg/kg of MA did not produce per se any effect on sleeping time ($P>.05$, Fig. 7a).

In contrast, no potentiation was found between these compounds in the holeboard (Fig. 7b) or in the plus maze (Fig. 7c) tests.

4. Discussion

Hölzl and Godau (1989) using a dichloromethane extract of *V. officinalis* and some of its fractions detected inhibition on the binding of ^{3}H-FNZ to the BDZ-bs. The specific activity was low and the reported figures were uncertain due to the complexity of the samples.

Mennini et al. (1993) made a valuable effort to characterize the interactions of crude extracts of *V. officinalis* and of two pure components in them with brain receptors. As expected, the results were suggestive but inconclusive; there was a clear inhibitory action at the BDZ-bs but it was not significant. Neither hydroxyvalerenic acid nor dihydrovaltrate exerted inhibition on the receptors tested.

The most recent contribution in this field was made by Bodesheim and Hölzl (1997) who were able to demonstrate that the lignan 1-hydroxypinoresinol, isolated from *V. officinalis*, is a ligand for the 5-HT$_{1A}$ serotonin receptor with an IC$_{50}$ ~2.5 μM. The lignan is inactive on the GABA receptors and on the BDZ-bs but has a low affinity (IC$_{50}$ ~25 μM) for the μ-opioid receptor. No in vivo effects of this compound were reported.

MA was the first compound isolated from valeriana that proved to have medium to high affinity for the BDZ-bs ($K_i = 0.5$ μM) (Wasowski et al., 2002) and, as described herein, is able to cause anxiolytic effects in

mice (Fig. 6b). Its concentration in *V. officinalis* and *V. wallichii* is approximately 60 μg/g of crude drug, but this apparently low value may still be therapeutically significant, if we consider that the pharmaceutical forms of valeriana, at the usual doses administered to humans, provide the equivalent of 3 g of crude drug, or more, per day.

Furthermore, the potentiating or synergic effect of MA plus HN (Fig. 7a) increases the probability of the significance of these flavonoids in human therapy.

HN is a novel compound in valeriana but a well-known substance in citrus. It was first described in 1828 by Lebreton and Brandeo, as reported by Higby (1941). The citrus industry has produced ample amounts of this flavanone glycoside for a long time (Higby, 1941).

In 1936, the laboratory of the discoverer of vitamin C, Albert Szent Györgyi, found that HN, together with other citrus flavonoids, was useful to treat abnormal capillary permeability and fragility associated with vitamin C deficiency (Rusznyák and Szent-Györgyi, 1936). From that time onwards, HN properties were intensively explored in many clinical and experimental conditions (Garg et al., 2001). Nevertheless, the remarkable activities of HN on the CNS, described here, eluded detection. The failure to discover its sedative (Fig. 5b) and sleep-inducing properties (Fig. 5c) may be probably explained by the fact that the drug generally used in the early experiments was the racemic variety provided by the citrus industry (Higby, 1941; Garg et al., 2001), while we found here that the CNS active isomer is the 2*S*(−) form. This is supported by the results in Table 1, where the sleep-inducing activity of the commercial drug is compared to that of the HN isolated from *V. wallichii*. Table 1 shows that the citrus HN is less active (w/w) than the valeriana HN. These in vivo results are in line with the analysis of the same samples performed by CD, which indicates that citrus HN contains approximately 60% of pharmacologically available S active form.

HN is not a ligand for the BDZ-bs, or to any of the receptors assayed in the experiments shown in Fig. 2; hence, the mechanism of its sedative and sleep-enhancing activities is still obscure.

MA is not a sleep-enhancing compound, as shown in Fig. 7a, but its joint injection with HN gives rise to a dramatic increase in the hypnotic effect (Fig. 7a). The weight relationship between HN and MA doses in these experiments is 2:1, but an almost similar potentiating effect was also obtained with a ratio 2:0.1 (not shown).

This apparently synergic enhancement further supports the relevance of MA and HN in the tranquilizing and sleep-inducing activities of valerianas.

MA and HN are now members of the growing family of natural flavonoids with activity on the CNS (Medina et al., 1997; Marder and Paladini, 2002), and their properties suggest that they are promising drug leads in the field.

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