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Pharmacological Profile of Apigenin, a Flavonoid Isolated from *Matricaria chamomilla*

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ABSTRACT. Dried flowers of *Matricaria chamomilla* L. are largely used to provide sedative as well as spasmolytic effects. In the present study, we examined in particular the pharmacological property of a fraction isolated from a methanolic extract of M. *chamomilla*, which was identified by HPLC–MS–MS analysis as apigenin. By radioreceptor binding assays, we demonstrated the ability of the flavone to displace a specific radioligand, [^{3}H]Ro 15-1788, from the central benzodiazepine binding site. Electrophysiological studies performed on cultured cerebellar granule cells showed that apigenin reduced GABA (gamma-aminobutyric acid)-activated Cl^- currents in a dose-dependent fashion. The effect was blocked by co-application of Ro 15-1788, a specific benzodiazepine receptor antagonist. Accordingly, apigenin reduced the latency in the onset of picrotoxin-induced convulsions. Moreover, apigenin injected i.p. in rats reduced locomotor activity, but did not demonstrate anxiolytic, myorelaxant, or anticonvulsant activities. The present results seem to suggest that the inhibitory activity of apigenin on locomotor behaviour in rats cannot be ascribed to an interaction with $GABA_A$–benzodiazepine receptor but to other neurotransmission systems, since it is not blocked by Ro 15-1788. BIOCHEM PHARMACOL **59**;11:1387–1394, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. *Matricaria chamomilla* L.; apigenin; benzodiazepine receptors; GABA-activated Cl^- currents; locomotor behaviour; rat

Bzs§ are the most widely prescribed drugs for the treatment of anxiety and sleep disturbances. It has been demonstrated that Bzs bind with high affinity to specific recognition sites located on an α subunit of the $GABA_A$ receptor, hence potentiating GABA-induced chloride currents [1–4]. In addition, a γ subunit is required for functional modulation of the channel by Bzs [5]. During the last 10 years, several Bzs and Bz-like ligands have been found in natural sources [6], food [7], and medicinal plants [8–11]. Authentic diazepam and nordiazepam have been identified in wheat and potato [7] and lentils and mushrooms [8], while flavonoids exerting Bz-like activity such as chrysin and apigenin were found in *Passiflora coerulea* and in *Matricaria chamomilla* L., respectively [9, 10]. The dried flower heads of M. *chamomilla* have been reported to exhibit spasmolytic and sedative properties, although the active components responsible for the sedative activity have not yet been fully characterised.

Hence, the aim of the present study was to examine in more detail the composition of M. *chamomilla*. Herein, we report the isolation and purification of a compound identified by HPLC–MS–MS analysis as apigenin, which was tested for its ability to modulate GABA-activated Cl^- currents and for its sedative, anxiolytic, and anticonvulsant properties.

MATERIALS AND METHODS

Extraction and HPLC Analysis

Twenty grams of dried flower head powder of M. *chamomilla* L. (Grosserbe) was suspended in 200 mL of methanol for 24 hr. The suspension, after filtration, was evaporated under vacuum. The residue was analysed by reverse-phase HPLC separation. The residue was reconstituted with 80% water/0.1% trifluoroacetic acid and 20% acetonitrile and chromatographed at 0.8 mL/min on a LiChrospher 100 RP-18 column (240 × 4.0 mm; 5 μM; Merck) equilibrated with 80% water/0.1% trifluoroacetic acid and 20% acetonitrile. Absorbance was monitored at 230 nm. The sample was chromatographed using a water/0.1% trifluoroacetic acid and acetonitrile gradient at 0.5%/min from 20 to 58% acetonitrile. Seventy-five fractions (one per minute) were collected, lyophilised, and reconstituted in water before the radioreceptor binding assay.

Central Benzodiazepine Receptor Binding

Aliquots of the reconstituted HPLC fractions were tested for their ability to inhibit [^{3}H]Ro 15-1788 (8-fluoro-3-

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§ *Abbreviations:* Bzs, benzodiazepines; and $GABA_A$, gamma-aminobutyric acid.

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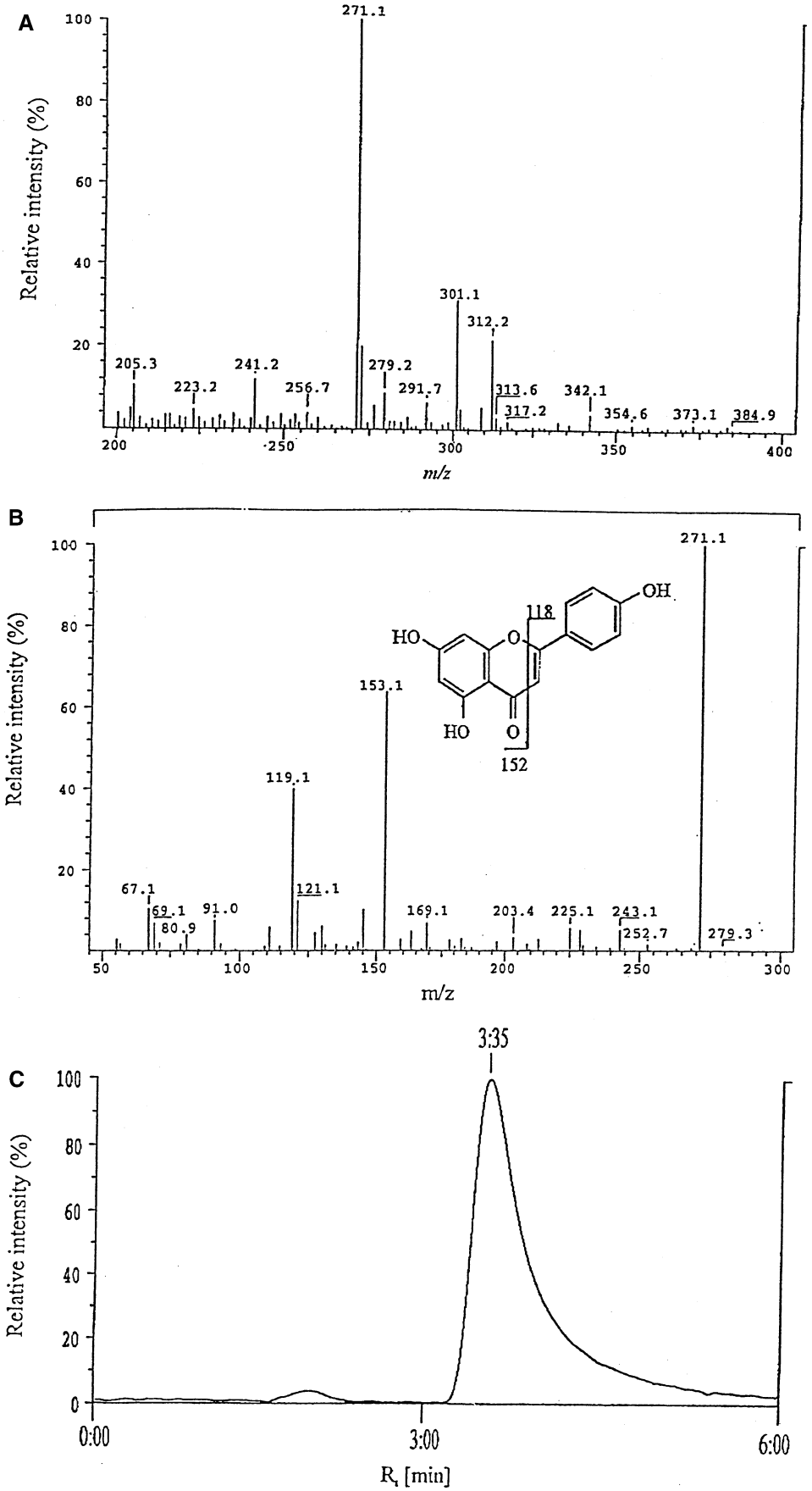

FIG. 1. (A) Electrospray (ESI) product ion mass spectrum of apigenin (MW = 270); precursor ion: *m/z* 271 representing the protonated apigenin; collision energy: 35 eV; collision gas: argon at 270 mPa. (B) Electrospray (ESI) mass spectrum of apigenin (MW = 270), showing the most abundant protonated molecular ion *m/z* 271. (C) HPLC–UV chromatogram of apigenin. Mobile phase: acetonitrile/water (0.1% trifluoroacetic acid) 50:50 (v/v); column: Kpauer LiChrospher 60 RP select B (100 × 2 mm i.d.; 5 μm); flow rate 200 μL/min; UV: 254 nm.

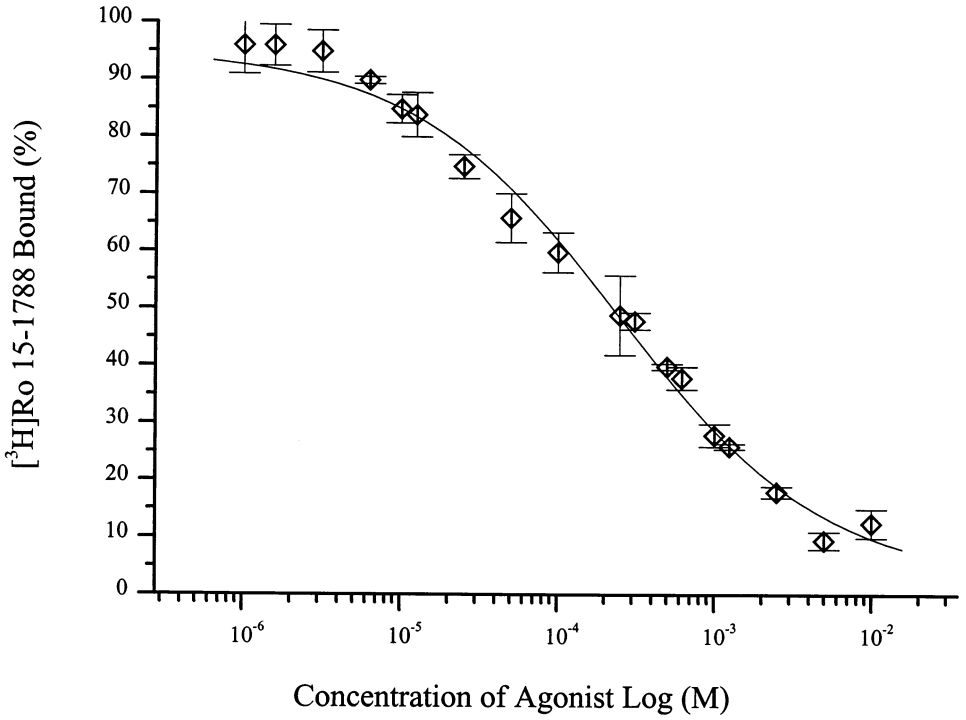

FIG. 2. Inhibition of [^{3}H]Ro 15-1788 specific binding by apigenin (10^{-2} M–10^{-6} M). Values reported are the means of three experiments ± SD.

carboethoxy-5,6-dihydro-5-methyl-6-oxo-4*H* imidazol [1,5-*a*] 1,4 benzodiazepine) specific binding to rat cerebellar membranes prepared as described previously [12]. Cerebellar rat membranes (100 μL, 500–1000 μg of protein/mL) resuspended in phosphate buffer pH 7.4 were incubated with 1 nM [^{3}H]Ro 15-1788 (25 μL) (specific activity 3.09 TBq/mmol; NEN™ Life Science Products) and aliquots (25 μL) of the reconstituted HPLC fractions or 1 μM unlabeled diazepam (Hoffmann-La Roche) to assess the extent of non-specific binding. The final volume (250 μL) was obtained by adding phosphate buffer (100 μL). The mixture was incubated at 0–4° for 1 hr. The reaction was terminated by vacuum filtration through glass fibre filters (Whatman GF/C) followed by 3 washings with 3 mL of ice-cold phosphate buffer. Radioactivity was determined in an LS 1701 RackBeta liquid scintillation counter with 3 mL of Ready Safe (Beckman) scintillation liquid. Protein concentration was determined by the method of Bradford [13] with BSA used as a standard. For the inhibition curves, different concentrations (10^{-2}–10^{-6}M) of apigenin (100 μL) (Sigma) were used. Binding data were analysed using the GraphPad Prism program.

HPLC–Electrospray–Tandem Mass Spectrometry Analysis

High performance liquid chromatography–electrospray ionisation–tandem mass spectrometry (HPLC–ESI–MS/MS) was utilised according to the method of Kleinschnitz *et al.* [14]. The analysis was performed using a triple-stage quadrupole TSQ 7000 LC–MS–MS system with electrospray interface (Finnigan MAT). Data acquisition and mass spectrometric evaluation were conducted on Personal DECstation 5000/33 (Digital Equipment) and ICIS 8.1 software (Finnigan MAT). For HPLC, an Applied Biosystem 140B Solvent Delivery System (Applied Biosystems) equipped with two 10-mL syringes and a LiChrospher 60-RP select B column (100 × 2.0 mm i.d., 5 μm) were used. Aliquots of the lyophilised samples were redissolved in 100 μL of methanol–water–acetonitrile (1:1:1 v/v/v). Separations were performed by using a linear gradient. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was acetonitrile. The gradient program was: 0–10 min 20–80% B. The loop injection was 5 μL and the solvent flow was set to 200 μL/min. The mass spectrometer was operated in the selected reaction monitoring mode (SRM) with argon at a pressure of 0.27 Pa as collision gas.

Primary Cultures of Cerebellar Granule Cells and Electrophysiological Recording

Primary culture of cerebellar granule neurons were prepared from 7–8-day-old Sprague–Dawley rats as previously described [15]. Briefly, cells from cerebella were dispersed with trypsin (0.24 mg/mL) (Sigma) and plated at a density of 10^6 cells/mL on 35-mm Falcon dishes coated with poly-L-lisine (10 μg/mL) (Sigma). Cells were grown in basal Eagle's medium (Irvine Scientific) supplemented with 10% fetal bovine serum (Hy Clone), 2 mM glutamine, and 100 μg/mL of gentamycin (Sigma) and maintained at 37° in 5% CO_2. Cytosine arabinofuranoside (10 μM) (Sigma) was added to the cultures 24 hr after plating to prevent astroglia proliferation. Recordings were performed on cerebellar neurons after 7 days in culture under the stage of an inverted microscope (Nikon Diaphot 300). The whole-cell configuration of the patch-clamp technique was employed [16]. Electrodes had a resistance of 5–7 MΩ when filled

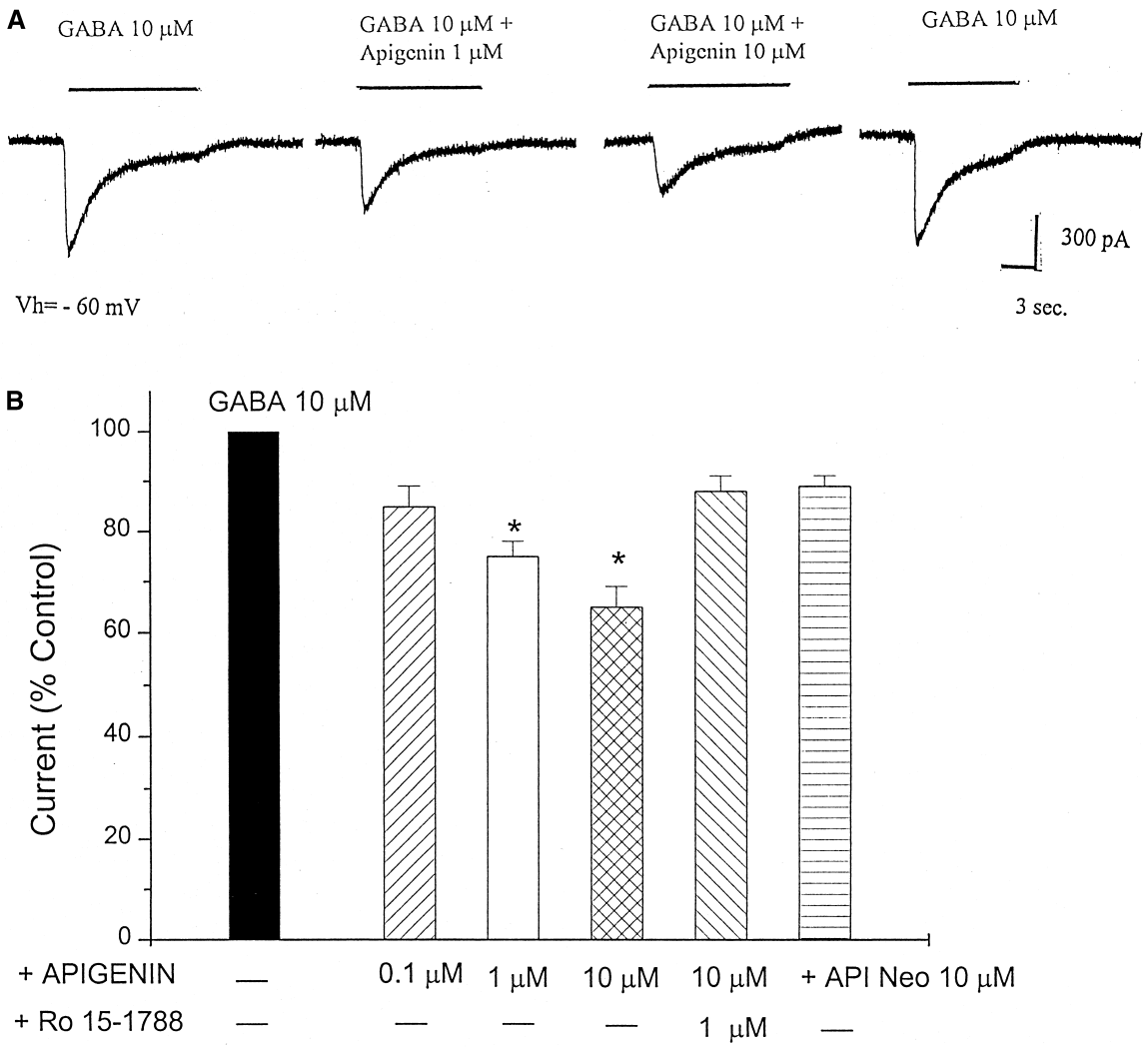

FIG. 3. A representative trace of GABA-activated Cl^- current modulated by 1 and 10 apigenin μM (A). The neuron was voltage-clamped at −60 mV and exposed to the drug for the time underlined by the bar. Histograms, (B), show the % of control current after the application of 0.1 = (N = 5), 1 (N = 8), and 10 μM apigenin (N = 10), 10 μM apigenin + 1 μM Ro 15-1788 (N = 0.07), and 10 μM apigenin 7-O-neohesperiodoside (API Neo). Each bar represents the mean ± SEM per group of cells. (N = 7). *P < vs GABA (ANOVA test).

with KCl internal solution. Currents were amplified with an Axopatch ID amplifier (Axon Instruments), filtered at 5 kHz, and digitised at 10 kHz by using pCLAMP software 6 (Axon Instruments). Data were analysed using the Clampex software (Axon Instruments). Intracellular solution contained (mM): KCl 140, $MgCl_2$ 3, EGTA 5, HEPES 5, ATP-Mg 2, pH 7.3 with KOH. Cells were continuously perfused with the external solution (mM): NaCl 145, KCl 5, $CaCl_2$ 1, HEPES 5, glucose 5, sucrose 20, pH 7.4 with NaOH. Apigenin and Ro 15-1788 were dissolved in DMSO at 10^{-2} M, diluted to a final concentration in the extracellular solution (final concentration DMSO 0.01%), and applied directly by gravity through a Y-tube perfusion system. GABA was dissolved in extracellular medium. As apigenin is usually conjugated in nature to sugars, the apigenin 7-O-neohesperidoside (Sigma) was also tested by dissolving it in extracellular solution and applying it directly as above.

Pharmacological Assays

Sprague–Dawley male rats (180–200 g b.wt.) were used throughout the experiments. They were housed in a climatised room with free access to food and water and maintained on a 12:12-hr light: darkness cycle. Apigenin was dissolved in DMSO (1 mL/kg) and injected i.p. 15 min before pharmacological tests, dosed from 0.5 to 50 mg/kg corresponding to 1–100 mM. Ro 15-1788 (3 mg/kg) were dissolved in DMSO and injected i.p. 15 min before apigenin. Diazepam, used as reference drug in the anxiolytic test, was injected i.p. (1 mg/kg) 20 min before the test.

The open field test was performed by placing each individual animal in the centre of a square arena (100 × 100 × 50 cm hr) with a black floor. Rats were continuously filmed for 10 min with a telecamera connected to a computerised system (Motion Analyser BM800) and the following parameters recorded: i) total length of the rat

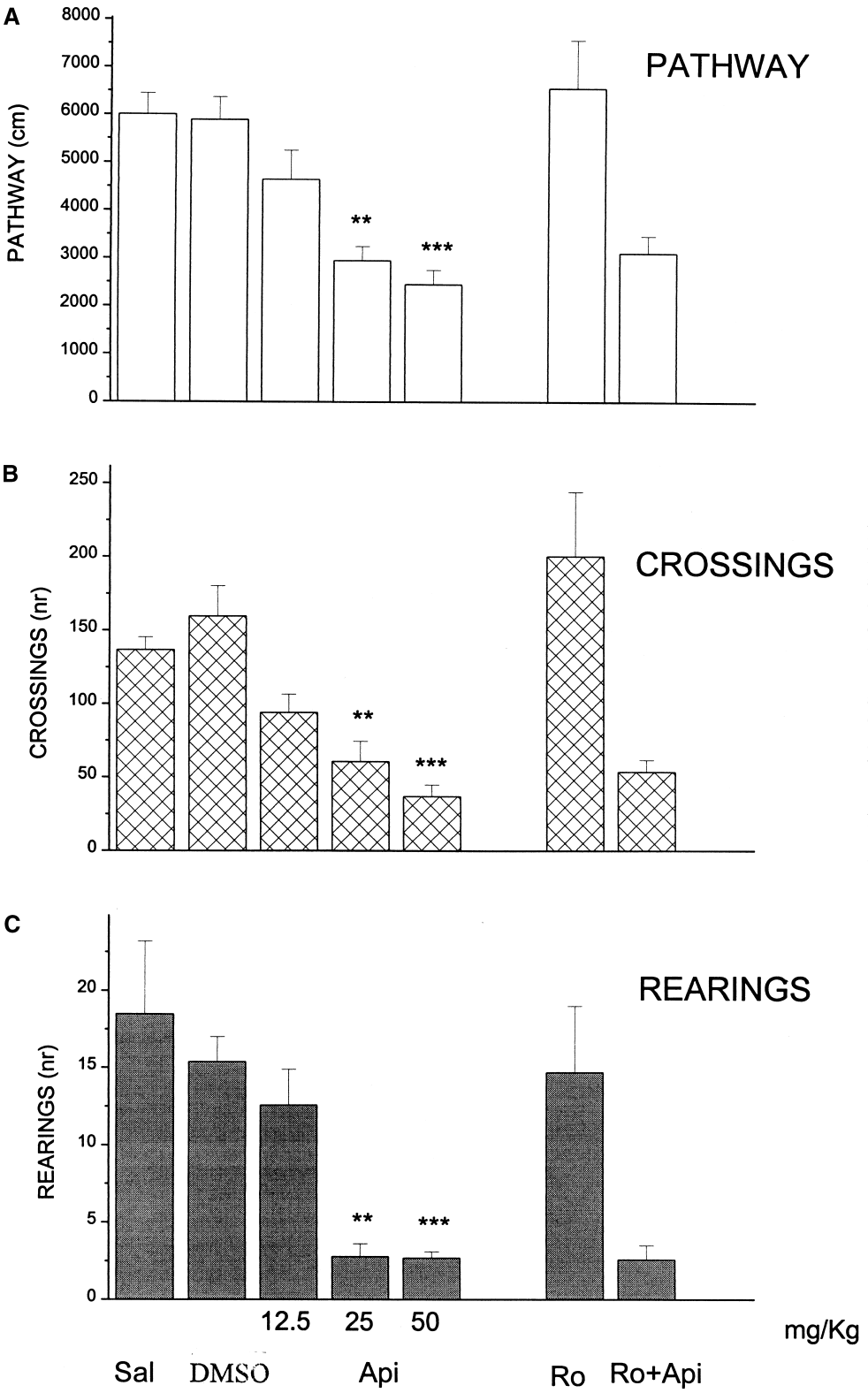

FIG. 4. Influence of apigenin on locomotor behaviour in rats, evaluated as total length of pathway (A), crossings (B), and rearings (C) during the open field test. Rats received Bz antagonist (Ro 15-1788, 3 mg/kg) 15 min before apigenin treatment (25 mg/kg). Control rats received saline (Sal) or vehicle (DMSO) 1 mL/kg. Each bar represents the mean ± SEM of 10 animals per group. Observation period: 10 min. ***$P < 0.001$, **$P < 0.01$ vs DMSO (ANOVA test).

pathway during ambulatory activity; ii) number of crossings of 10 × 10-cm squares; and iii) time spent in immobility. A "blind" observer measured, by an external video, the number of times the rat reared upon its hind feet (rearings). In addition, the system provided the route pattern for each rat.

Seizures were induced by the s.c. injection of two doses of picrotoxin (6 and 8 mg/kg). Apigenin was administered i.p. 15 min prior to picrotoxin. The following parameters were recorded: i) number of rats with seizures (%); ii) latency, i.e. the time between the injection and the onset of the first jerk or clonus; iii) death rate; and iv) death latency. The seizures were scored according to Zhang *et al.* [17] as follows:

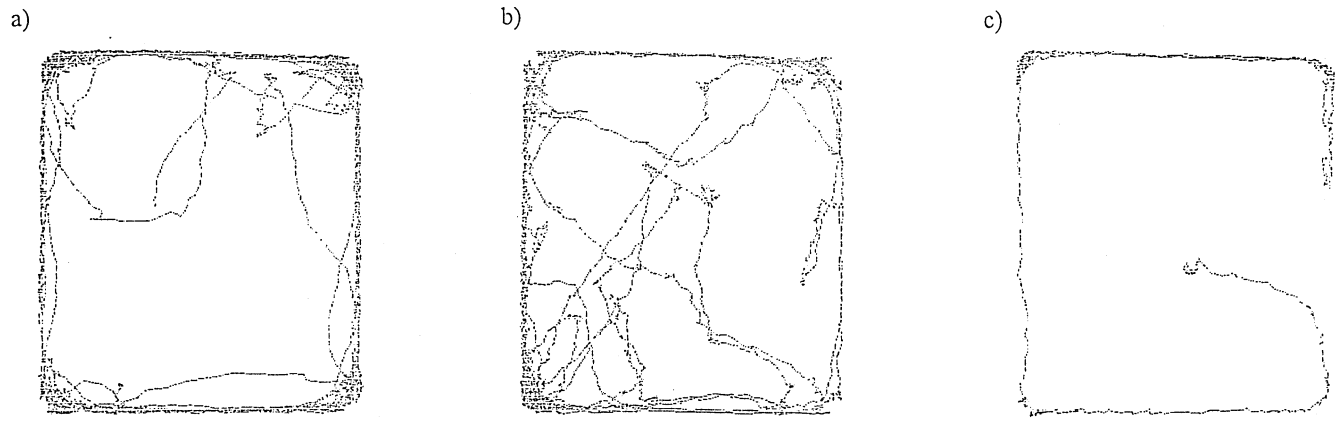

FIG. 5. Typical pattern routes of rats from different experimental groups in open field: a) saline 1 mL/kg; b) DMSO 1 mL/kg; c) apigenin 50 mg/kg.

0 (no seizure), 1 (jerks), 2 (clonus without tonic extension), and 3 (tonic-clonic seizure).

The elevated plus-maze consisted of two open (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm h) with an open roof, arranged such that the two arms of each type were opposite each other. The maze was elevated to a height of 50 cm. Following the procedure described by Pellow *et al.* [18], rats were placed individually in the centre of the maze and were observed for 5 min, during which the number of entries and the time spent in the open and closed arms of the maze were measured. After each trial, the apparatus was cleaned with an odoriferous solution.

In the horizontal wire test, the ability of rats to grasp a horizontal wire placed 30 cm above the laboratory table was determined as previously described [19].

RESULTS

Isolation of Apigenin and Binding Experiments

HPLC analysis of the methanol extract of *M. chamomilla* revealed the presence of several compounds able to bind central benzodiazepine receptors which are still under identification procedure. One of the compounds was identified by HPLC–ESI–MS/MS analysis as apigenin, as determined by comparing the spectra shown in Fig. 1 with those of synthetic apigenin. The inhibition curve of apigenin reported in Fig. 2 showed the ability of apigenin to inhibit [^{3}H]Ro 15-1788 specific binding to rat cerebellar membranes with a very low affinity ($IC_{50} = 2.5 \times 10^{-4}$ M; Hill slope = −0.47).

Electrophysiological Experiments

Apigenin decreased GABA-activated Cl^- currents measured in primary culture of cerebellar granule cells in a dose-dependent way. Cl^- currents evoked by 10 μM GABA were reduced by 15 ± 3% (mean ± SEM, N = 5) when apigenin was applied at 0.1 μM and were signficantly reduced by 24 ± 2% (N = 8) and 32 ± 4% (N = 10) when apigenin was applied at 1 and 10 μM, respectively. Ro 15-1788 at 1 μM was able to antagonise the effect of 10 μM apigenin but, when applied alone, was ineffective on GABA-evoked currents. The effect of apigenin 7-*O*-neohesperidoside (10 μM) on Cl^- currents was not different from control (Fig. 3).

Pharmacological Assays

The locomotor activity of rats administered with apigenin was compared with that of rats treated with DMSO (Fig. 4). The injection of DMSO failed to induce a relevant effect on the different parameters used to analyse the locomotor behaviour in rats in comparison with saline-injected rats. The total distance travelled by rats was significantly reduced by apigenin injection dosed at 25 and 50, but not at 12.5, mg/kg in comparison with DMSO administration. Moreover, there was a significant reduction in the number of crossings and rearings in apigenin-treated rats (25 and 50 mg/kg) in comparison with vehicle-injected rats. In an attempt to explain the sedative activity of apigenin in terms of a Bz-like effect, we pretreated the apigenin-injected rats with Ro 15-1788, a central Bz antagonist. The sedative effect elicited by apigenin was not affected by Ro 15-1788 pretreatment. Furthermore, when injected alone, Ro 15-1788 did not exert any significant effect on the measured behavioural parameters. In the open field test, the time spent in immobility was not affected by apigenin (data not shown). The routes reported in Fig. 5 represent a typical pattern of pathways of rats treated with apigenin or vehicle in comparison with controls. The pathway travelled by a rat treated with apigenin (Panel c) was shorter than that of a rat treated with saline (Panel a) or with vehicle (Panel b).

In the seizures test, apigenin (25 and 50 mg/kg) injected 15 min before picrotoxin (6 and 8 mg/kg) significantly reduced the time of latency in the onset of convulsions, but failed to affect the other parameters (Table 1).

Apigenin, dosed from 0.5 to 10 mg/kg, was tested in the elevated plus-maze using diazepam, injected i.p. at the dose of 1 mg/kg, as reference compound. The results reported in

TABLE 1. Effect of apigenin on picrotoxin-induced seizures in rats

Pretreatment (mg/kg)	Treatment (mg/kg)	Rats with seizures (%)	Seizure latency (min)	Seizure score	Death rate (%)	Death latency (min)
DMSO	picrotoxin 6	100	21.7 ± 1.6	2.0 ± 0.2	8.3	60.1 ± 7.3
apigenin 25	picrotoxin 6	100	16.5 ± 1.7*	1.9 ± 0.4	8.3	62.0 ± 8.1
DMSO	picrotoxin 8	100	24.6 ± 1.7	2.6 ± 0.2	37.5	80.0 ± 8.9
apigenin 50	picrotoxin 8	100	17.7 ± 1.8*	3.0 ± 0	50	83.5 ± 9.5

Data are expressed as means ± SEM, obtained from groups of 8 rats each. Apigenin was injected i.p. 15 min before picrotoxin s.c.
*$P < 0.05$ vs corresponding values of picrotoxin-treated rats (Mann–Whitney U test).

Fig. 6 demonstrate that apigenin did not exert any anxiolytic effect. It must, however, be underlined that apigenin, when dosed at 1 mg/kg, did increase the number of entries and the time spent in the open arms, but the data did not reach statistical significance when compared with vehicle-treated rats (Mann–Whitney U test). Moreover, we did not observe any increase in the number of entries or the time spent in the open arms after administration of lower and higher doses of apigenin. Diazepam, on the other hand, showed a clear anxiolytic effect under the same experimental conditions.

In the horizontal wire test, performed to evaluate a myorelaxant effect, apigenin injected up to 50 mg/kg did not affect rat-grasping behaviour.

DISCUSSION

In traditional medicine, the dried flower heads of *M. chamomilla* are widely used to obtain sedative, spasmolytic, and anti-inflammatory effects. Furthermore, a depressive activity of a lyophilised infusion of flowers of *M. chamomilla* on the central nervous system was demonstrated in mice by Della Loggia *et al.* [20]. Many studies have been performed with the aim of clarifying which component is responsible for the sedative effect. Viola *et al.* [10] demonstrated that *M. chamomilla* contains several Bz receptor ligands, the most interesting of which, from a pharmacological point of view, is apigenin. The authors reported that apigenin exhibits anxiolytic activity in mice without any sedative or myorelaxant effects.

Our *in vitro* experiments showed that apigenin inhibited the binding of [^{3}H]Ro 15-1788, a specific ligand for central Bz receptors, with an IC_{50} of 2.5×10^{-4} M. In our opinion, the high value of the IC_{50} and the negative Hill indicate a very low affinity of apigenin for these binding sites. In electrophysiological experiments, we have demonstrated that apigenin, but not apigenin 7-*O*-neohesperidoside, was able to reduce GABA-activated chloride currents. This effect was blocked by Ro 15-1788, suggesting a selective

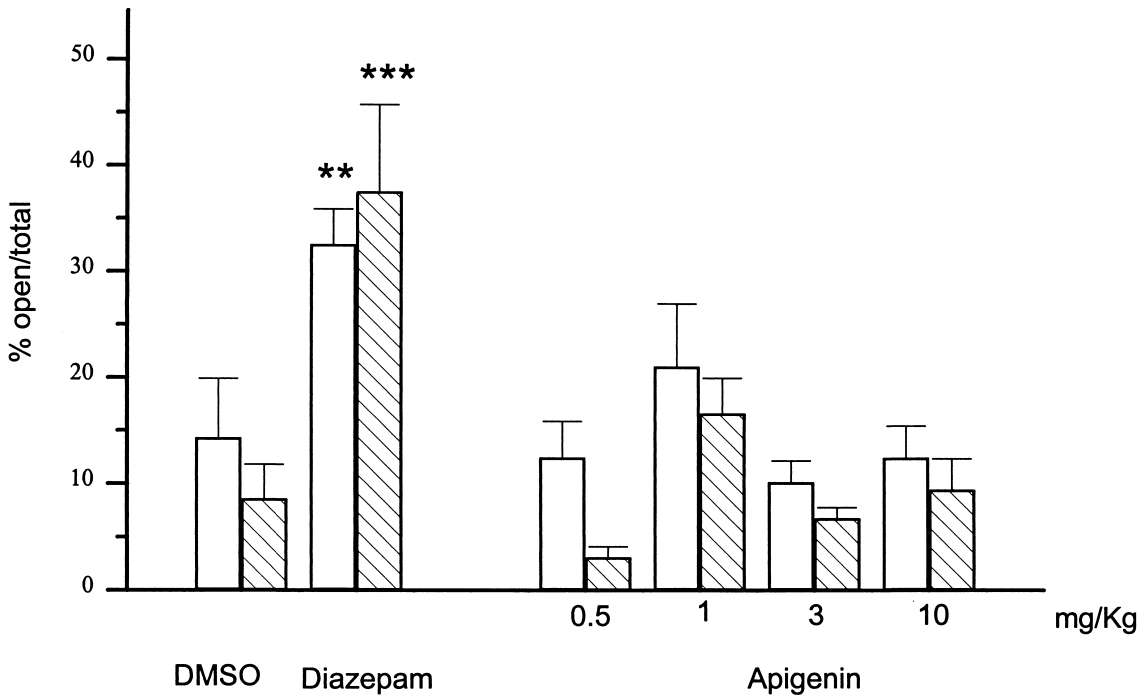

FIG. 6. Influence of apigenin on rat behaviour in elevated plus-maze test. Mean percentage (± SEM) of open arm entries (white bars) and of time spent in the open arms (hatched bars) in rats given a 5-min test in the elevated plus-maze, 15 min after i.p. injection with DMSO (1 mL/kg) or apigenin (0.5, 1, 3, and 10 mg/kg) or 20 min after diazepam (1 mg/kg). Number of animals per group = 8. ***P < 0.001, **P < 0.01 vs DMSO (ANOVA test).

activity at the $GABA_A$ receptor level. The ability of apigenin to reduce GABA activity *in vitro* could account for the *in vivo* results which showed that apigenin decreased the latency of seizures measured in picrotoxin-injected rats.

From the behavioural point of view, apigenin injected i.p. in adult male rats exerts sedative properties, without exhibiting a clear-cut anxiolytic and myorelaxant effect. When tested in the open field, apigenin-treated rats showed a significant reduction in locomotor activity in comparison with vehicle-treated rats. The depressive effect, however, was not reverted by pretreatment with central Bz antagonist, thus suggesting that the sedative property of apigenin is not related to a direct effect on Bz receptors. Moreover, experiments performed in order to evaluate the ability of apigenin to potentiate the pentobarbital sleeping time in rats did not demonstrate any effect (data not shown).

All together, the present data seem to indicate that, in contrast to the results reported by Viola *et al.* [10] and Salgueiro *et al.* [21], apigenin does not exert any anxiolytic effect. We think that the sedative effect of apigenin is not mediated by Bz receptors since: a) the affinity of apigenin is very low; b) apigenin decreased GABA-activated Cl^- currents, suggesting an anxiogenic rather than an anxiolytic effect; and c) the sedative effect is not blocked by Bz-specific antagonist.

As a consequence, the sedative activity of apigenin cannot be ascribed to an activation of $GABA_A$ receptors, and this effect will be better characterised by studying its interaction on different neurotransmission systems other than $GABA_A$. From these data, we can surmise that the sedative effect of *M. chamomilla* extracts can be ascribed to other compounds with Bz-like activity as previously reported by us [11], more than to apigenin itself.

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