J. Pharm. Pharmacol. 1999, 51: 519–526
Received September 14, 1998

Flavonoids and the Central Nervous System: from Forgotten Factors to Potent Anxiolytic Compounds

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Abstract

The list of activities of plant flavonoids did not include effects on the central nervous system (CNS) up to 1990, when our laboratory described the existence of natural anxiolytic flavonoids. The first of these was chrysin (5,7-dihydroxyflavone), followed by apigenin (5,7,4′-trihydroxyflavone) and flavone itself. Semisynthetic derivatives of flavone obtained by introducing halogens, nitro groups or both in its molecule, give rise to high affinity ligands for the benzodiazepine receptor, active in-vivo; 6,3′-dinitroflavone, for example, is an anxiolytic drug 30 times more potent than diazepam.

The data collected in this paper make clear that some natural flavonoids are CNS-active molecules and that the chemical modification of the flavone nucleus dramatically increases their anxiolytic potency.

Flavonoids comprise an important class of secondary metabolites in plants. More than 5,000 different flavonoids have been described and the properties of many of them studied (Harborne 1994). Their chemical structure is based on the phenylchromane or flavane ring system (Figure 1).

The biological and pharmacological properties of flavonoids cover a wide spectrum of actions. A rather conservative list, limited to mammals, include the following effects: antioxidative, anti-allergic, anti-inflammatory, antitoxic, hepato-protective, inhibition of many enzymes, anti-carcinogenic. For a review see Middleton & Kandaswami (1994), but very few, if any, actions on the central nervous system (CNS) of mammals were known before 1989.

Our research, which eventually contributed to filling this gap, started with an investigation of the neural basis of anxiety (De Robertis et al 1988; Medina et al 1993).

Anxiety is defined as a subjective emotional state of uneasiness, not pleasant and even fearful. When the anxiety reaches pathological levels the subject experiences conductual changes, apprehension, motor troubles, sweating, and hypertension (Pratt 1992).

Traditional medicine has many cures for this ailment, most of them based on herbal preparations, but also modern medicinal chemistry has provided several drugs which are more or less effective, for the same purpose. The most spectacular success was achieved in 1957 with the synthesis of the benzodiazepines (Figure 2) (Sternbach 1978), which still are, after 40 years of intense clinical research and use, the best treatments for anxiety.

Benzodiazepines, however, also produce several side-effects, including sedation, muscle relaxation, alcohol incompatibility, amnesia and addiction (Woods et al 1992). These drawbacks have to be carefully considered in clinical application.

Several neurotransmitter receptor systems have been involved in the neurobiological mechanisms that regulate anxiety. Among others we may mention the receptors for cholecystokinin, excitatory aminoacids, serotonin (5-hydroxytryptamine) and noradrenaline (Hamon 1997). A large proportion of these neurotransmitter receptor systems are reciprocally controlled by the principal inhibitory system operating in the brain, the gamma amino butyric acid receptor ($GABA_A$ receptor), which is activated by the homonymous transmitter.

*Presented by Alejandro C. Paladini at the British Pharmaceutical Conference, September 9, 1998, Eastbourne

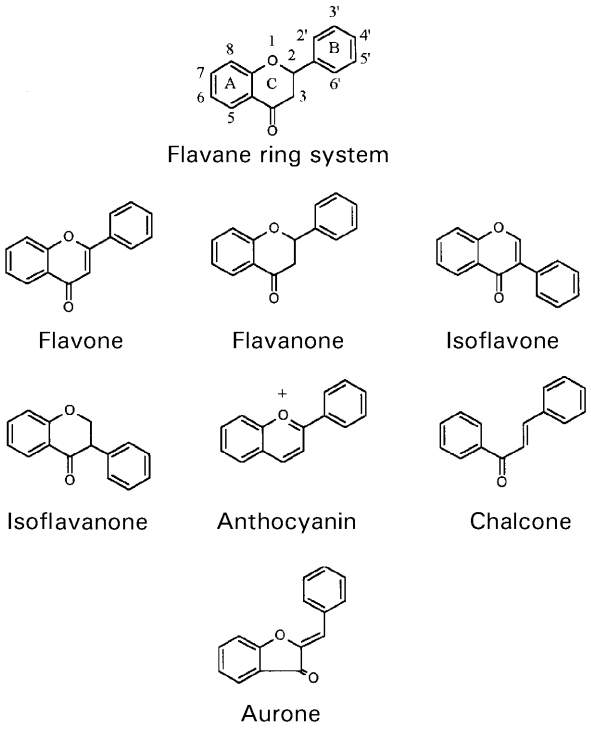

Figure 1. Basic structures of the flavonoids. The flavane ring system consists of a C6 C3 unit (ring B and carbons 2,3 and 4) and a C6 unit (ring A). The flavonoids are subdivided according to the positions of the phenyl ring B, into flavanes (2-phenyl), isoflavanes (3-phenyl) and neoflavanes (4-phenyl). Depending on the degree of oxidation of the pyran ring, they are classified as flavones, flavanones, isoflavones, isoflavanones and anthocyanins. For structural and biochemical reasons, the chalcones and aurones are also considered to be flavonoids.

The $GABA_A$ receptor is a chloride ion-channel receptor that allows the entry of these ions into the neurons under the influence of gamma amino butyric acid. In general terms, this causes a hyperpolarization that decreases the possibility of generating nerve impulses.

Anxiolytic benzodiazepines, inactive by themselves, bind to an area of the $GABA_A$ receptor different to that recognizing GABA itself. This site, which is called the benzodiazepine receptor (Mohler & Okada 1977; Squires & Braestrup 1977), increases the action of GABA under the influence of the benzodiazepines.

The $GABA_A$ receptor is a pentameric complex formed by isoforms of 5 different subunits (McKernan & Whiting 1996) giving rise to numerous variants of the main receptor and accordingly, of the benzodiazepine receptor within. This emerging diversity is being intensively studied at present at the molecular level but previous research indicates the existence of at least two pharmacological groups of benzodiazepine-receptor subtypes, type I and type II, not homogeneously

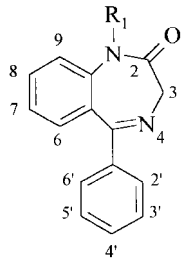

Figure 2. General formula of 1,4-benzodiazepines. Diazepam is the 7-chloro-1-methyl derivative. Flunitrazepam is the 2′-fluoro-1-methyl-7-nitro derivative.

distributed in the CNS (Siegharth & Karobath 1980; Trifiletti et al 1984; Niddam et al 1987; Mohler et al 1995; McKernan & Whiting 1996).

Although benzodiazepines are laboratory products they were recently found also in nature and, appropriately, their first detection was in the mammalian brain (Sangameswaran et al 1986). They were then identified in many other sources including foods, rumen, plasma and cow and human milk (Medina & Paladini 1993).

When we attempted detection of benzodiazepines in several plants, including some used to prepare tranquillizing infusions, we unexpectedly discovered that some flavonoids present in them, were ligands for the benzodiazepine receptors (Medina et al 1989).

This finding led to the isolation, from the medicinal plant *Passiflora coerulea*, of the flavonoid chrysin (Table 2), which proved to be a competitive ligand for the benzodiazepine receptors with a K_i value of 3 μM. (Table 1) (Medina et al 1990).

The literature registers only two previous instances of flavonoids as ligands for the benzodiazepine receptors: the isoflavans equol and 7,3′-dihydroxyisoflavan (Luk et al 1983), and the biflavonoid amentoflavone (Nielsen et al 1988) (Table 2). These compounds are inactive in-vivo while chrysin, is anxiolytic in mice at an intraperitoneal dose of 1 mg kg^{-1} (Figure 3, Table 1) (Wolfman et al 1994).

The animal models described in the literature to study anxiety have in common the use of fear to mimic the human syndrome. In our studies we have used a sensitive test in mice monitoring a fear-avoidance conflict whereby an animal chooses to enter either closed or open-sided arms of an elevated maze in the shape of a plus. It has been demonstrated that treatment of mice with a clinically effective anxiolytic drug results in an increase in the number and time spent by the animals in the open-sided arms of the maze (Pellow et al 1985; Pellow & File 1986a, b; Lister 1987).

Table 1. Biochemical parameters and pharmacological effects in mice of CNS-active natural flavonoids.

Compound	Biochemical parameters[a]		Pharmacological effects in mice (minimum effective intraperitoneal dose, mg kg^{-1})				
	K_i (μM)	GABA ratio	Anxiolytic[b]	Sedative[c]	Myorelaxant[d]	Locomotor[e]	Hypnotic[f]
Chrysin	3	1·4	1	NE (up to 10)	NE (up to 30)	+(0·6)	ND
Apigenin	3	1·4	3	30	NE (up to 100)	−(100)	ND
Flavone	1	1·4	3	NE (up to 10)	NE (up to 10)	−(1)	ND
Cirsiliol	200	ND	ND	2	ND	ND	4
Diazepam	0·007	2	0·03	1	1	+ (0·6), −(1)	3

[a][^{3}H]Flunitrazepam binding to rat cerebral cortical synaptosomal membranes (Levi de Stein et al 1989; Medina et al 1983), [b]elevated plus-maze test (Pellow et al 1985; Pellow & File 1986a, b; Lister 1987), [c]holeboard test (File & Pellow 1985), [d]wire test (Bonetti et al 1982), [e]locomotor activity (Wolfman et al 1994),[f]pentobarbital-induced sleep tests (Anca et al 1992;Wada et al 1993). Diazepam values are shown for comparison. + = increase, − = decrease, ND = not determined, NE = no effect.

Table 2. Affinity of natural flavonoids for the benzodiazepine receptors.

Name	Structure	K_i (μM)
	Monoflavonoids	
Flavone	2-Phenylchromone	1[a]
Norwogonin	5,7,8-Trihydroxyflavone	0·88[b]
Dinatin	5,7,4′-Trihydroxy-6-methoxyflavone	1·3[c]
Chrysin	5,7-Dihydroxyflavone	3[d]
Apigenin	5,7,4′-Trihydroxyflavone	3[e]
Tangeretin	5,6,7,8,4′-Pentamethoxyflavone	2·9[b]
Baicalein	5,6,7-Trihydroxyflavone	4[b]
Isoquercitrin	5,7,3′,4′-Tetrahydroxyflavone-3-O-Glc	10
Skrofulein	5,4′-Dihydroxy-6,7-dimethoxyflavone	23[c]
Eriodictyol	5,7,3′,4′-Tetrahydroxyflavanone	> 25[b]
Naringenin	5,7,4′-Trihydroxyflavanone	> 25[b]
Genistein	5,7,4′-Trihydroxyisoflavone	> 25[b]
Phloretin	4,2′,4′,6′-Tetrahydroxydihydrochalcone	> 25[b]
Flavanone		> 40[a]
	7,3′-Dihydroxyisoflavan	45[f]
Tectochrysin	5-Hydroxy-7-methoxyflavone	50[a]
Equol	7,4′-Dihydroxyisoflavan	80[f]
Kaempferol	3,5,7,4′-Tetrahydroxyflavone	93[g]
Galangin	3,5,7-Trihydroxyflavone	> 100[a]
Luteolin	5,7,3′,4′-Tetrahydroxyflavone	> 100[g]
Fisetin	3,7,3′,4′-Tetrahydroxyflavone	> 100[g]
Quercetin	3,5,7,3′,4′-Pentahydroxyflavone	> 100[g]
Rutin	5,7,3′,4′-Tetrahydroxyflavone-3-O-Glc-Rha	> 100
Rhoifolin	5,4′-Dihydroxyflavone-7-O-Glc-Rha	> 100
Myricetin	3,5,7,3′,4′,5′-Hexahydroxyflavone	> 100
Morin	3,5,7,2′,4′-Pentahydroxyflavone	> 100[g]
Cirsiliol	5,3′,4′-Trihydroxy-6,7-dimethoxyflavone	> 100[h]
	5,7-Dimethoxyflavone	> 100[a]
Acacetin	5,7-Dihydroxy-4′-methoxyflavone	> 100[a]
Orientin	5,7,3′,4′-Tetrahydroxyflavone-8-Glc	ND[i]
Isoorientin	5,7,3′,4′-Tetrahydroxyflavone-6-Glc	ND[i]
	Biflavonoids	
Amentoflavone	I-4′, II-4′, I-5, II-5, I-7, II-7-Hexahydroxy [I-3′, II-8] biflavone	0·007[j]
Ginkgetin	II-4′, I-5, II-5, II-7-Tetrahydroxy-I-4′, I-7-dimethoxy [I-3′, II-8] biflavone	2–6
Isoginkgetin	I-5, II-5, I-7, II-7-Tetrahydroxy-I-4′, II-4′-dimethoxy [I-3′, II-8] biflavone	2–6
Bilobetin	II-4′, I-5, II-5, I-7, II-7-Pentahydroxy-I-4′-methoxy [I-3′, II-8] biflavone	> 5
Sciadopitysin	I-5, II-5, II-7-Trihydroxy-I-4′, I-7, II-4′-trimethoxy [I-3′, II-8] biflavone	> 5

The K_i values estimate the inhibition of [^{3}H]diazepam or [^{3}H]flunitrazepam binding to rat cerebral cortical synaptosomal membranes (Levi de Stein et al 1989; Medina et al 1983). The K_i values without a reference are unpublished results from our laboratory. ND = K_i not determined but anxiolytic effects detected. Flavanone has not been reported free in nature (Harborne 1994).[a] Marder et al 1996b, [b]Ai et al 1997, [c]Shen et al 1994, [d]Wolfman et al 1994, [e]Viola et al 1995, [f]Luk et al 1983, [g]Viola et al 1994, [h]Marder et al 1996a, [i]Okuyama et al 1996, [j]Nielsen et al 1988.

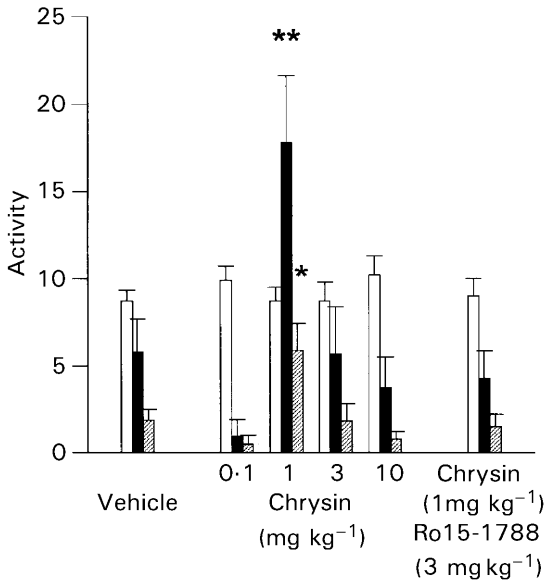

Figure 3. Anxiolytic activity of chrysin. The figure shows the performance of mice during a 5-min period in the elevated plus-maze test 20 min after intraperitoneal injection with vehicle, chrysin or chrysin (1 mg kg^{-1}) preceded 7 min before by Ro 15-1788 (3 mg kg^{-1}). Results are expressed as mean ± s.e.m. of the number of total arm entries (□), percentage of open arm entries (■) and percentage of time (s) spent in the open arms (▨). $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ compared with controls (Dunnett's multiple comparison test applied after analysis of variance). Number of animals in each group ranged between 15 and 22 (adapted from Wolfman et al 1994).

The results obtained in the plus-maze with intraperitoneal injections of chrysin unambiguously indicate its anxiolytic action through the activation of benzodiazepine receptors because the prior administration of Ro 15-1788, a well known antagonist for the benzodiazepine receptors (Bonetti et al 1982), abolishes this effect (Figure 3).

When chrysin was tested in other animal models measuring its effect on sedation (holeboard test, File & Pellow 1985), muscle relaxation (wire test, Bonetti et al 1982) or locomotion (Opto Varimex apparatus, Wolfman et al 1994), it was found that all these functions were much less affected by this flavonoid in comparison with diazepam (Table 1).

All the properties described for chrysin, as well as its GABA ratio of 1.4 (Table 1) support the contention that this flavonoid is a partial agonist of the benzodiazepine receptors. The GABA-ratio (ratio between K_i values obtained in the absence or in the presence of 100 μM GABA in the binding assay (Braestrup et al 1983; Sanger 1985; Chan & Farb 1985), is a biochemical parameter widely used to characterize the intrinsic activity of benzodiazepine-receptor ligands. GABA increases the binding affinity of benzodiazepine agonists (GABA-ratio > 1), has no effect on benzodiazepine-receptor antagonists (GABA-ratio $\cong$ 1) and decreases the affinity of benzodiazepine-receptor inverse agonists (GABA- ratio < 1). Partial agonists have GABA-ratios close to 1·5.

Further research in our laboratory permitted the isolation and identification of apigenin, another anxiolytic flavonoid, from *Matricaria recutita* (Viola et al 1995); the discovery of flavone as an anxiolytic ligand of the benzodiazepine receptors (Marder et al 1996b; Medina et al 1997) and the isolation and identification of cirsiliol, from *Salvia guaranítica* (Marder et al 1996a) (Table 2). Cirsiliol is a low affinity ligand of the benzodiazepine receptors, with partial selectivity for the type I benzodiazepine receptors possessing hypnotic and sedative properties (Table 1) (Viola et al 1997b).

The existence of flavonoids active on the CNS prompted a survey of the benzodiazepine receptor binding properties of several natural compounds of this class (Table 2). Most of the natural flavonoids therein have very low or no affinity at all for the benzodiazepine receptors. Although the sample is small, the trend towards no-affinity is clear and it may explain the prevailing consensus in the field that flavonoids were not relevant neuroactive compounds.

As is usual in medicinal chemistry, we attempted to improve the properties of the natural active compounds by chemical modifications. The addition of electronegative atoms such as halogens and nitro groups suggested itself after the demonstration of their effectiveness in increasing the activity of the benzodiazepines (Haefely et al 1985).

The majority of the chemical modifications attempted in our laboratory were made on flavone or in hydroxy- or methoxy-derivatives of this molecule. From the results collected in Table 3, it is clear that carbon atoms 6 and 3′ in the flavone nucleus are the most effective positions to place electronegative substituents, while position 3, for instance, seems to be deleterious for affinity.

A methyl group in carbon 6 is also effective in increasing the affinity of flavone for the benzodiazepine receptors (compare the respective K_i values in Tables 2 and 3). Recent experiments in our laboratory have also shown that the introduction of a bromine atom in position 3′ of 6-methyl flavone increases further its affinity; the K_i value of 6-methyl-3′-bromo flavone is approximately 16 nM (n = 4).

Restricting our analysis to some of the ligands in Table 3 with more affinity for the benzodiazepine receptors, we may comment on the properties of the various compounds as follows.

Table 3. Affinity of synthetic flavonoids for the benzodiazepine receptors.

Compound	K_i (μM)	Compound	K_i (μM)
6-Bromo-3′-nitroflavone	0·001 and 0·016[a]	5-Hydroxyflavone	1·3[f]
6-Chloro-3′-nitroflavone	0·008[b]	4′-Hydroxyflavone	2·1[f]
6-Bromo-3′-chloroflavone	0·017[c]	6-Bromo-4′-methoxyflavone	2·10[c]
6,3′-Dibromoflavone	0·019[b]	3′-Methoxyflavone	2·4[c]
6-Chloro-3′-bromoflavone	0·023[c]	6-Fluoro-3′-methoxyflavone	2·5[c]
6,3′-Dichloroflavone	0·023[c]	6,4′-Difluoroflavone	2·5[c]
6-Nitro-3′-bromoflavone	0·025[b]	6-Methyl-3′,4′-dimethoxyflavone	3·4[f]
6,3′-Dinitroflavone	0·026[d]	3′-Fluoroflavone	3·55[c]
6-Bromo-3′-fluoroflavone	0·042[c]	4′-Fluoroflavone	3·6[c]
6-Bromoflavone	0·070[e]	6-Fluoroflavone	4·5[b]
6-Bromo-4′-fluoroflavone	0·115[c]	7-Hydroxyflavone	5·3[f]
6-Chloro-3′-fluoroflavone	0·117[c]	6,7-Dihydroxyflavone	6·8[f]
6-Methylflavone	0·120[f]	5,4′-Dihydroxyflavone	7·3[f]
6-Chloroflavone	0·164[b]	2′-Fluoro-5,7-dihydroxyflavone	8[e]
6-Fluoro-3′-nitroflavone	0·180[b]	2′-Chloro-5,7-dihydroxyflavone	8[e]
6-Chloro-4′-fluoroflavone	0·181[c]	6,2′-Dinitroflavone	> 10[b]
6-Fluoro-3′-chloroflavone	0·199[c]	6,4′-Dinitroflavone	> 10[d]
6-Bromo-4′-nitroflavone	0·200[a]	4′-Methoxyflavone	> 10[c]
6-bromo-2′-nitroflavone	0·208[a]	6-Fluoro-4′-methoxyflavone	> 10[c]
6-Fluoro-3′-bromoflavone	0·236[c]	6-Chloro-4′-bromoflavone	> 10[c]
6-Nitroflavone	0·275[b]	6,4′-Dibromoflavone	> 10[c]
3′-Nitroflavone	0·285[b]	2′-Fluoroflavone	> 10[c]
6-Bromo-2′-fluoroflavone	0·377[c]	6,2′-Difluoroflavone	> 10[c]
3′-Bromoflavone	0·413[b]	4′-Nitroflavone	> 10[b]
6-Chloro-2′-fluoroflavone	0·417[c]	6-Fluoro-4′-nitroflavone	> 10[c]
6-Hydroxyflavone	0·580[f]	6-Chloro-4′-nitroflavone	> 10[c]
6-Bromo-3′-methoxyflavone	0·609[c]	4′-Bromoflavone	> 10[b]
3′-Chloroflavone	0·614[b]	6-Fluoro-4′-bromoflavone	> 10[c]
6,8-Dibromo-5,7-dihydroxyflavone	0·700[e]	2′-Nitroflavone	> 20[b]
6-Chloro-3′-methoxyflavone	0·848[c]	3′,5′-Dinitroflavone	> 20[b]
6-Methoxyflavone	0·860[f]	3-Bromo-3′-nitroflavone	> 20[b]
6,3′-Difluoroflavone	0·920[c]	3-Bromoflavone	> 75[e]
6,8-Diiodo-5,7-dihydroxyflavone	1[e]	6,3-Dibromoflavone	> 75[e]
6-Chloro-4′-methoxyflavone	1·25[c]		

The K_i values estimate the inhibition of [^{3}H]flunitrazepam binding to rat cerebral cortical synaptosomal membranes (Levi de Stein et al 1989;Medina et al 1983). The chemistry applied in our laboratory was based on the Baker-Venkataraman transformation for synthesizing flavones (Marder et al 1997, 1998).[a]Viola et al 1997a, [b]Marder et al 1997, [c]Marder et al 1998, [d]Marder et al 1995, [e]Marder et al 1996b, [f]Ai et al 1997.

6-Bromo-3′-nitroflavone (Viola et al 1997a)

6-Bromo-3′-nitroflavone exhibits a different affinity for subtypes I and II of benzodiazepine receptors with $K_i = 1$ and 16 nM, respectively. Its GABA ratio of 1·38 suggests that it is a partial agonist. The plus-maze test reveals that it is anxiolytic at intraperitoneal doses between 10 and 300 μg kg^{-1}.

6-Chloro-3′-nitroflavone (unpublished results)

6-Chloro-3′-nitroflavone has no anxiolytic effects. The GABA ratio of 1·16 suggests antagonistic properties. This is supported by direct pharmacological experiments showing that it cancels anxiolysis, anticonvulsion and amnesia caused by diazepam.

6,3′-Dibromoflavone and 6-nitro-3′-bromoflavone (unpublished results)

The pharmacological profiles of both compounds suggests that they are partial agonists. They produce anxiolytic effects at intraperitoneal doses ranging from 0·1 to 3 mg kg^{-1}, depending on the compound. Neither compound is myorelaxant or anticonvulsant, but at 30 mg kg^{-1} both are sedative.

6-Bromoflavone (Marder et al 1996b)

6-Bromoflavone is a full agonist with a GABA ratio of 1·6–2·0. It causes anxiolytic effects in mice at intraperitoneal doses between 0·5 and 3 mg kg^{-1} and also produces sedation and myorelaxation at doses starting at 3 and 10 mg kg^{-1}, respectively.

6,3′-Dinitroflavone (Marder et al 1995; Wolfman et al 1996)

6,3′-Dinitroflavone is the most active anxiolytic derivative of flavone prepared in our laboratory (Figure 4). It is, at least, 30 times more potent by weight than diazepam and 3000 times more potent than flavone, or apigenin (Figure 5). It also has a very favourable separation index (Table 4). The separation index is the ratio between the minimal

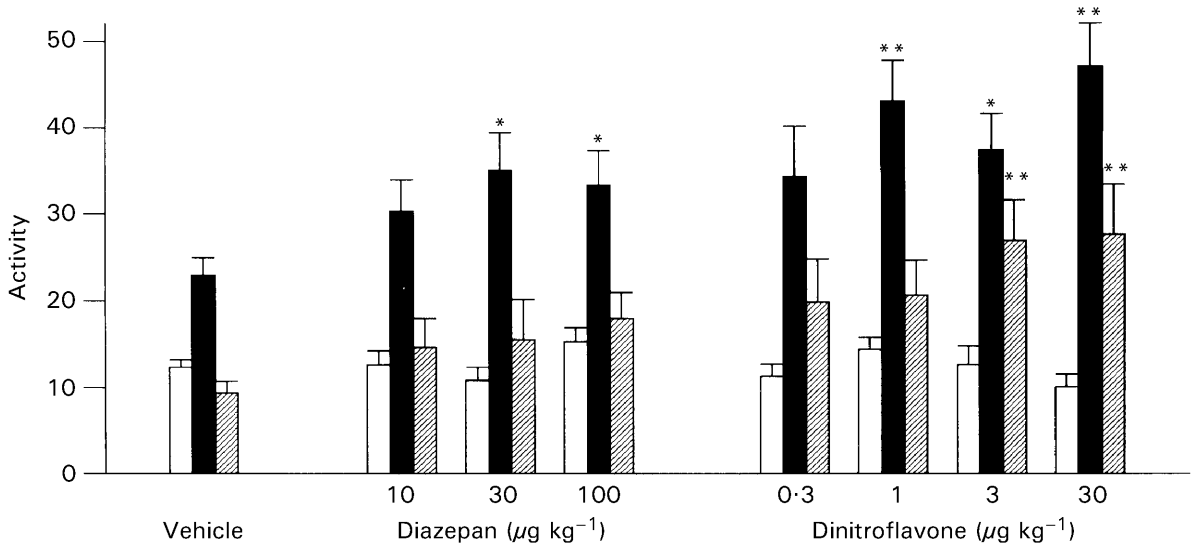

Figure 4. Anxiolytic activity of 6,3′-dinitroflavone, compared with that of diazepam. The figure shows the performance of mice during a 5-min period in the elevated plus-maze test, 15 min after intraperitoneal injection of vehicle, dinitroflavone or diazepam. Results are expressed as in Figure 3. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ compared with vehicle (Dunnet's multiple comparison test). Number of animals in each group ranged between 9 and 16 (adapted from Marder et al 1995).

sedative dose and the maximal anxiolytic dose. Its magnitude qualifies the pharmacological selectivity of the compound. 6,3′-Dinitroflavone, which also is active by the oral route, as shown in Figure 6, is the most promising candidate to become a useful drug in the therapy of anxiety.

The data collected in this paper make clear that a number of natural flavonoids are CNS-active molecules and that the chemical modification of the flavone nucleus dramatically increases its affinity for the benzodiazepine receptors and its anxiolytic potency.

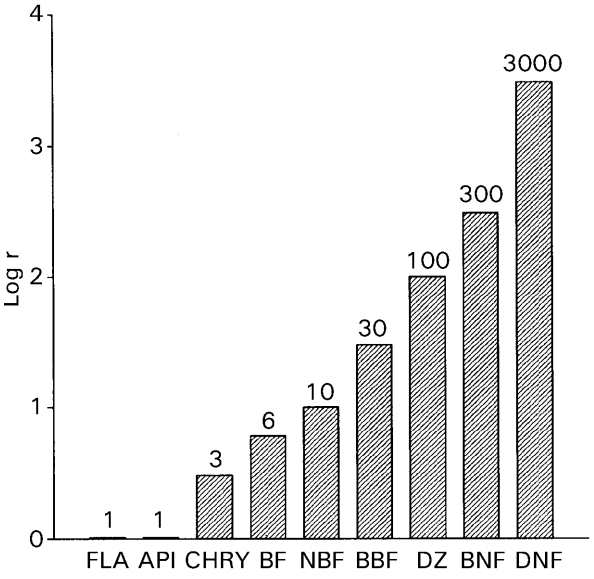

Figure 5. Anxiolytic potency of apigenin, chrysin and various synthetic flavonoids expressed as the logarithm of the ratio (r) of the minimum anxiolytic dose of flavone (by weight), to the corresponding value for each compound. FLA = flavone, API = apigenin, CHRY = chrysin, BF = 6-bromoflavone, NBF = 6-nitro-3′bromoflavone, BBF = 6,3′-dibromoflavone, BNF = 6-bromo-3′-nitroflavone, DNF = 6,3′-dinitroflavone, DZ = diazepam, included as a reference. The numbers on top of the bars are the decimal values of the ratios.

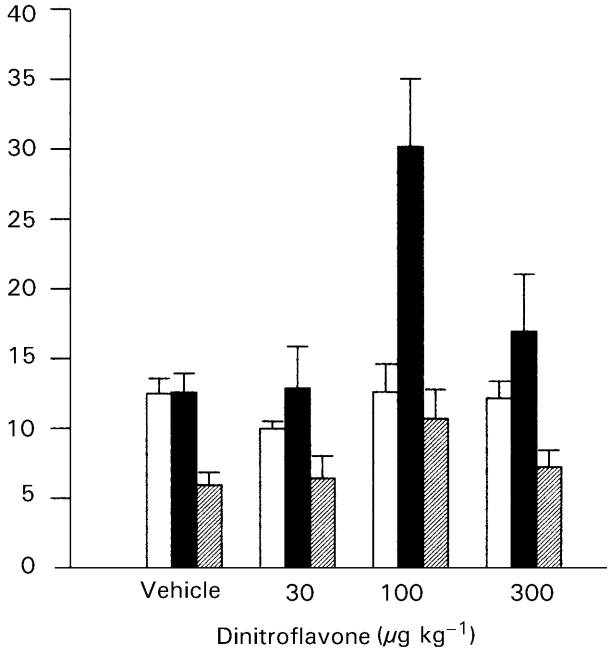

Figure 6. Anxiolytic activity of 6,3′-dinitroflavone administered by the oral route. The figure shows the performance of mice during a 5-min period in the elevated plus-maze test 35 min after oral administration of vehicle or dinitroflavone. Results are expressed as in Figure 3. $*P < 0{\cdot}01$ significantly different from controls (Dunnett's multiple comparison test). Number of animals in each group ranged between 12 and 20.

Table 4. Separation indexes.

Compound	Separation index
6-Bromoflavone	1
Diazepam	3
Apigenin	10
6-Nitro-3′-bromoflavone	10
6-Bromo-3′-nitroflavone	100
6,3′-Dibromoflavone	100
6,3′-Dinitroflavone	300

The separation index is the ratio between the minimal sedative dose and the maximal anxiolytic dose.

The explanation of these results in molecular terms is not yet available but a window has been opened onto the field of anxiety pharmacology and many possibilities may now be explored, either by synthetic chemistry or molecular pharmacology.

It is concluded that flavonoids are no longer forgotten factors for the CNS. Their role has been discovered and their medicinal potentials brought into focus.

Acknowledgements

This work was supported by the National Research Council (Argentina), the University of Buenos Aires, the International Foundation for Science (Sweden), the Organization of American States, and Strathclyde Institute for Drug Research, University of Strathclyde, Glasgow.

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