

# The Flavonoid Glycosides, Myricitrin, Gossypin and Naringin Exert Anxiolytic Action in Mice

Sebastian P. Fernandez · Michael Nguyen ·  
Tin Thing Yow · Cindy Chu · Graham A. R. Johnston ·  
Jane R. Hanrahan · Mary Chebib

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**Abstract** The consumption of flavonoid-rich foods, in particular fruits and vegetables, has been epidemiologically associated with a reduced risk of heart disease, neurodegenerative disease, cancer and other chronic diseases. Flavonoid glycosides, the main class of flavonoids, have been shown to exert CNS-mediated activities, particularly as sedative-hypnotics, analgesics or both, nevertheless no studies have evaluated these agents in anxiety. This study assessed the potential anxiolytic effect of three flavonoid glycosides, myricitrin, naringin and gossypin, in the elevated plus maze test (EPM). Myricitrin (1 mg/kg) was effective on the EPM showing a clear anxiolytic effect with no signs of sedation. However, higher doses showed possible sedative and myorelaxation effects. Gossypin and naringin both shared a similar profile, with low doses (1 mg/kg) inducing a robust anxiolytic effect which diminished with increasing doses of the flavonoids. Higher doses of these two flavonoids showed a dramatic increase in the open arm exploration accompanied by a decrease in locomotor activity. Hence, naringin (30 mg/kg) and gossypin (30 mg/kg) induce both anxiolytic and sedative effects. These results suggest that flavonoid glycosides have the potential to exert a range of CNS-mediated biological activities.

**Keywords** Naringin · Gossypin · Myricitrin · Flavone · Flavonoid glycosides · Elevated plus maze

## Introduction

The general term flavonoids is often used to classify a family of natural compounds, highly abundant in all higher plants, that have received significant therapeutic interest in recent years. Chemically, flavonoids possess a C6–C3–C6 basic skeleton modified by varied hydroxylation, methoxylation, glycosylation arrangements and combinations thereof, making it possible to classify them in more than ten subclasses that include flavones, flavonols, flavanones, anthocyanidins, isoflavones, chalcone, etc. Consequently, their chemical diversity is immense and over 5,000 naturally-occurring variations have been described. Perhaps related to the abovementioned diversity, flavonoids have been described to possess a wide range of biological activities such as antioxidant, anti-inflammatory, oestrogenic, cytotoxic, antitumoral, antiviral [1, 2]. Interestingly, the consumption of flavonoid-rich foods, in particular fruits and vegetables, has been epidemiologically associated with a reduced risk of heart disease, neurodegenerative disease, cancer and other chronic diseases, probably due to their activity as antioxidants and free radical scavengers [2]. Furthermore, clinical trials have provided evidence of the efficacy of some flavonoid-based preparations in the treatment of bone loss, vascular diseases and cancer [3–5].

Flavonoid glycosides are the main type in which flavonoids are found in nature. Glycosylation can occur at different positions and the number and nature of the carbohydrate units attached varies considerably. Importantly, glycosylation greatly influences chemical and physical properties altering in vivo bioavailability and

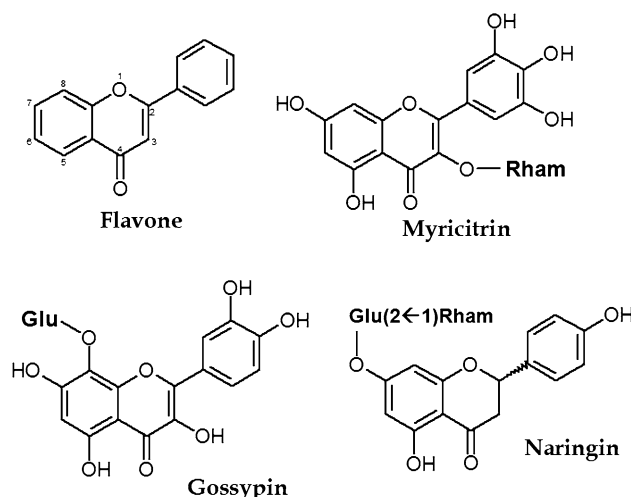
S. P. Fernandez · G. A. R. Johnston  
Faculty of Medicine, Department of Pharmacology,  
The University of Sydney, Sydney, NSW 2006, Australia

M. Nguyen · T. T. Yow · C. Chu · J. R. Hanrahan ·  
M. Chebib (✉)  
Faculty of Pharmacy, The University of Sydney, Sydney,  
NSW 2006, Australia  
e-mail: maryc@pharm.usyd.edu.au

possible pharmacological actions. Indeed, flavonoid glycosides have been shown to exert CNS-mediated activities, particularly as either sedative-hypnotics, analgesics or both. For example, the flavonoid glycosides hesperidin, linarin, naringin, diosmin, neohesperidin and gossypin have been reported to induce CNS depressant action in mice [6]. Furthermore, gossypin [7], myricitrin [8], linarin [9] and hesperidin [10] were shown to possess antinociceptive effects after systemic administration. The subsequent studies of the mechanism(s) of action accounting for these central effects have produced varied explanations where these compounds interact with diverse receptors, enzymes and ion channels.

Surprisingly, the action of flavonoid glycosides on anxiety levels has not been extensively explored, despite the fact that literature provides rich evidence that the neuronal mechanisms mediating anxiolysis, sedation and antinociception often converge. First, opioids are clinically known for their potent analgesic action, but they can also cause deep depression of the CNS and potentiate the sedative-hypnotic effects of benzodiazepines [11]. Moreover, preclinical evaluation of morphine has shown that this opioid agonist can induce anxiolysis after both peripheral and central administration [12, 13]. Second, it is well accepted that the opioid endogenous system plays a role in the anxiolytic action of benzodiazepines. Several studies have shown that opioid antagonists can block the anxiolytic responses to benzodiazepines and barbiturates [14]. Also, delta opioid receptor knockout mice showed an anxiogenic phenotype when compared to wild type in several behavioural tests [15]. Third, inhibitory GABAergic and glycinergic neurons in the dorsal horn of the spinal cord control the transmission of nociceptive signals from the periphery to the CNS, and some benzodiazepines have been shown to exert weak antinociceptive actions [16]. Interestingly, experiments conducted in GABA<sub>A</sub> point-mutated knock-in mice have revealed this GABAergic pain control in the spinal cord is mediated by receptors containing a  $\alpha 2$  subunit, the same subtype that controls anxiolytic and myorelaxant responses of benzodiazepines [16]. Finally, potentiation of morphine-induced analgesia has been described after co-injection with the benzodiazepine alprazolam [17] and the sedative flavonoid glycoside hesperidin [10].

In view of this evidence, we decided to evaluate the potential anxiolytic effect of three flavonoid glycosides, namely myricitrin, naringin and gossypin, in the elevated plus maze (EPM) test. Based on previous pharmacological studies, flavonoids were selected for their activity as analgesic (myricitrin), sedative (naringin) or both (gossypin; Fig. 1). We also tested the action of flavone, which represents the backbone of many flavonoids; thus bringing some insights into the role of the sugar residue. Behavioural evaluation was completed by assessing changes in



**Fig. 1** Molecular structure of flavone, myricitrin, gossypin and naringin. Except for flavone, flavonoids are glycosylated at different positions by distinct sugars. Naringin presents a chiral centre at position C2 allowing two possible configurations of this molecule. The sample used in this work correspond to a racemic mixture

spontaneous locomotor activity and muscle tension using the actimeter and the elevated wire tests, respectively. All three flavonoids exerted anxiolytic responses at doses below 3 mg/kg ip, without altering locomotor activity. However, anxiolysis diminished with increasing doses and significant sedative effects were detected for naringin and gossypin at doses of 30 and 60 mg/kg ip, respectively, but not for myricitrin. None of these compounds seemed to induce a significant myorelaxant action.

These results provide the first evidence that flavonoid glycosides exerting anxiolytic action on mice, and further strengthens the hypothesis that these dietary compounds enter the CNS after systemic administration.

## Experimental Procedure

### Materials

Commercial naringin, flavone and gossypin (purity  $\geq 90\%$ ) were purchased from Sigma–Aldrich Co. (St. Louis, Missouri, USA). Myricitrin dihydrate from Indofine Chemical Company Inc. (Hillsborough, New Jersey, USA). Diazepam was purchased from Apin Chemicals Ltd (Oxon, UK). Male Swiss mice were obtained from Animal Resources Centre (Perth, WA, Australia). Drug solutions for injection were prepared using DMSO and Tween 80 from Sigma.

### Animal Use

All procedures involving animals were in accordance with the Australian code of practice for the care and use of

animals for scientific purposes and were approved by the animal ethics committee of the University of Sydney.

Adult male Swiss mice (6–8 weeks-old) weighing 30–35 g were used in the behavioural animal models. All animals were maintained in a controlled environment (temperature 20–23°C; relative humidity 50–60%) and kept on a 12-h:12-h day/night cycle with light onset at 6:00 am. The animals were fed on normal mouse chow which contained flavonoids from plant sources. It is worthy to note that in a normal diet, not including herbal teas, food sources do not appear to provide sufficient amount of effective flavonoids for overt CNS effects [6]. Behavioural experiments were conducted from 10:00 am to 4:00 pm. Mice were housed undisturbed for at least 1 week before testing to negate the effects of shipping stress.

Diazepam and the flavonoids were dissolved for injection by the sequential addition of 5% DMSO, 20% of a 0.25% solution of Tween 80, and 75% saline. The mice were injected via ip route, 20 min before the behavioural tests. The volume of the ip injections was 0.15 ml/30 g of body weight, and 26-gauge needles were used. In each session, a control group receiving only vehicle was tested in parallel to those animals receiving drug treatment.

#### Elevated Plus Maze

The EPM consisted of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm). A raised ledge (3 mm high; 1 mm thick), constructed from clear Plexiglass, surrounded the perimeter of the open arms. The maze was arranged so that arms of the same type were opposite each other, connected by an open central platform (5 × 5 cm). The EPM was constructed from black Plexiglass and mounted on a base, elevating the maze 40 cm from ground level. Two 40 Watt incandescent light bulbs illuminated the experimental room. All sessions were videotaped by a camera positioned above the maze.

Each mouse was placed on the central platform, facing an open arm, and allowed to freely explore the apparatus for 5 min. Between animals, the apparatus was carefully cleaned with wet paper towel (mixture of ethanol, detergent and water) to remove any residue or odours. The exploratory behaviour was video recorded and later scored using ODLog software Version 2.3 for Windows (Macropod Software, Armidale, Australia). Four parameters of behaviour were measured: (1) number of entries in open arms, (2) number of entries in closed arms, (3) time spent in open arms (seconds), and (4) time spent in closed arms (seconds). An individual entry was recorded when all four paws of the mouse were on the arm. The % of open arm entries and % of time spent in the open arms were recorded as measure of anxiety state, while the number of closed entries was recorded as an indicator of locomotor activity [18, 19].

#### Locomotor Activity Assay (Actimeter)

Spontaneous locomotor activity was measured in the actimeter immediately after mice completed the EPM. The actimeter is a box made of Plexiglass, with a 45 × 21 cm floor and 21 cm high walls. On the walls and along the long axis of the box, 16 infrared emitting diodes and 16 infrared detectors were arranged in perfectly aligned pairs. Each of these pairs formed a movement sensor. The distance of the sensors was 3 cm from the floor of the box. The sensor interruptions measure animal activity along the long axis of the actimeter. The interruption of a sensor and the time of this event was detected and recorded by a personal computer running a Visual Basic program developed specifically for this apparatus. The data was shown in real time to the operator and, at the end of the experiment, all the information was automatically stored in a file for post-experiment analysis. Locomotor activity was expressed as total light beam breaks per 5 min [18].

#### Horizontal Wire Test

Muscle relaxant effects were assessed using the horizontal wire test. The apparatus consisted of a horizontally strung steel wire (1 mm in diameter; 15 cm long) suspended 20 cm above the table. Mice were lifted by the tail, allowed to grasp the wire with their forepaws, and then released. Two training sessions, performed at 10 min intervals, were carried out to assess the animals for their normal reactivity to the apparatus. Only animals capable of grasping the wire with their hind limbs within 5 s were included for testing, which was conducted the following day. The number of mice that failed the test was determined. Animals that fell from the wire or were unable to reach the wire with at least one hind paw within 5 s were considered to have failed the test [20].

#### Statistical Analysis

Results are expressed as mean ± the standard error of the mean (SEM), except for the horizontal wire test which was expressed as the percentage of mice grasping the wire. Data from the EPM and the locomotor activity assay were initially assessed using the D'Agostino and Pearson omnibus normality test and Bartlett's test for equal variances. One-way analysis of variance (ANOVA) was then used to compare several mice treatments. Subsequent post-hoc comparisons between individual treatments and controls were made using Dunnett's multiple comparison test when appropriate. Data from the horizontal wire test was analysed by Fisher's exact tests. In all cases, values with  $P < 0.05$  were considered statistically significant. All analyses were carried out using GraphPad Prism v5 for

Windows (GraphPad Software, San Diego, California, USA).

## Results

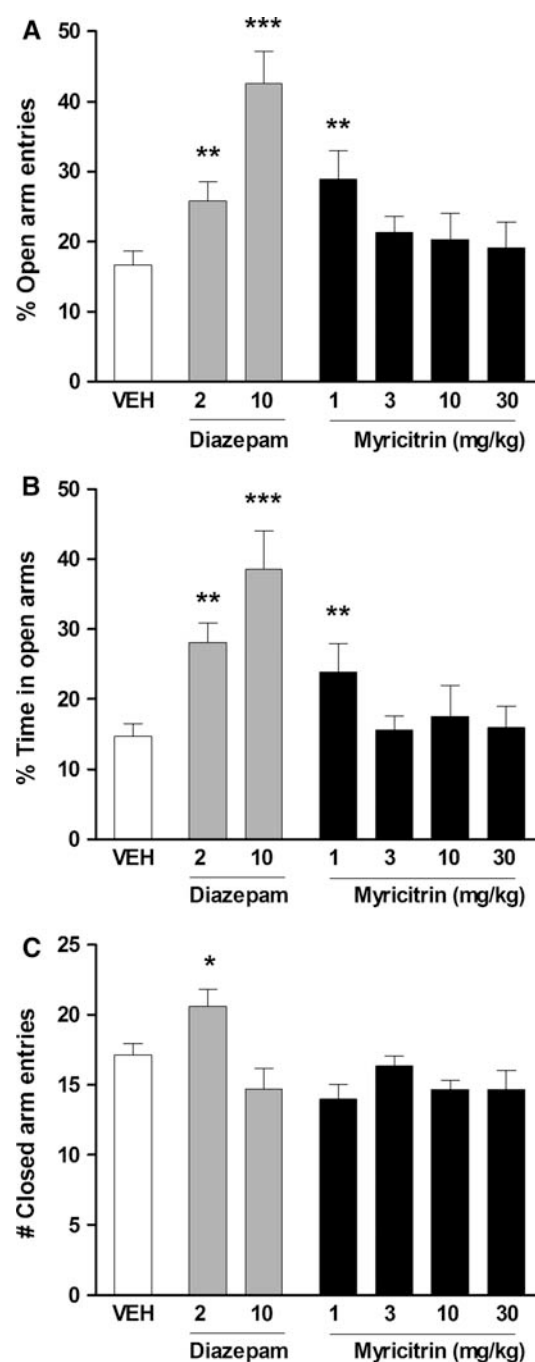
### Elevated Plus Maze Test

The effects of diazepam in the plus maze test are displayed in Figs. 2, 3, 4, 5 for comparison. Diazepam 2 mg/kg and 10 mg/kg were used as positive control groups. Both doses produced a significant increase in the % of open arm entries (2 mg/kg:  $P < 0.01$ ; 10 mg/kg:  $P < 0.001$ ) and the % of time spent in the open arms (2 mg/kg:  $P < 0.01$ ; 10 mg/kg:  $P < 0.001$ ) compared with the vehicle-treated group. In contrast, the number of closed arm entries was only significantly increased at a dose of 2 mg/kg ( $P < 0.05$ ).

Figure 2 shows the effects of myricitrin in the EPM. Myricitrin 1 mg/kg significantly increased the % of open arm entries ( $P < 0.01$ , Fig. 2a) and the time spent in the open arms ( $P < 0.01$ , Fig. 2b). This effect appeared to decrease as the dose of myricitrin was increased as no significant differences in open arm parameters were detected for the other doses of myricitrin when compared to vehicle. In addition, the number of closed arm entries was not significantly affected at any of the doses examined (Fig. 2c).

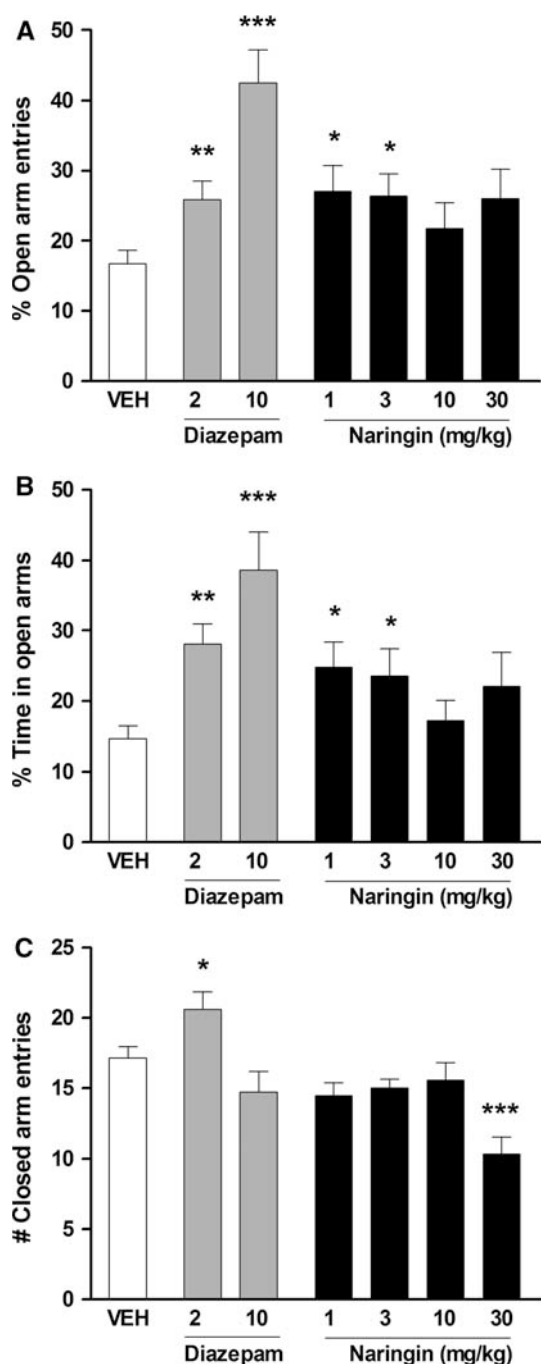
Figure 3 shows the effects of commercial naringin on the various parameters of the EPM. Naringin at doses of 1 and 3 mg/kg significantly increased the % of open arm entries ( $P < 0.05$ , Fig. 3a) and the # of time in the open arms ( $P < 0.05$ , Fig. 3b). Interestingly, a dose of 10 mg/kg of naringin was without effect but when injected at 30 mg/kg, naringin again increased these parameters but the differences failed to reach statistical significance. However, doses of 30 mg/kg of naringin also significantly reduced the number of closed arm entries compared to vehicle ( $P < 0.001$ , Fig. 3c), while all other doses of naringin did not significantly affect the number of closed arm entries.

The effects of gossypin in the EPM are shown in Fig. 4. Gossypin, at doses of 1, 3 and 10 mg/kg significantly augmented the % of open arm entries ( $P < 0.05$ , Fig. 4a), while the time spent in the open arms was significantly increased by low doses only (1 mg/kg:  $P < 0.05$ ; 3 mg/kg:  $P < 0.01$ , Fig. 4b). This effects were accompanied with no significant changes in the number of closed arm entries ( $P > 0.05$ , Fig. 4c). Similarly to naringin, gossypin actions on the exploration of open arms were decreasing with the dose (up to 30 mg/kg), but when the highest dose of 60 mg/kg was reached the % of open arm entries and time in open arms were significantly increased again ( $P < 0.01$ , Fig. 4a, b). Nevertheless, this dose also significantly decreased the number of entries to the enclosed arms ( $P < 0.001$ , Fig. 4c).



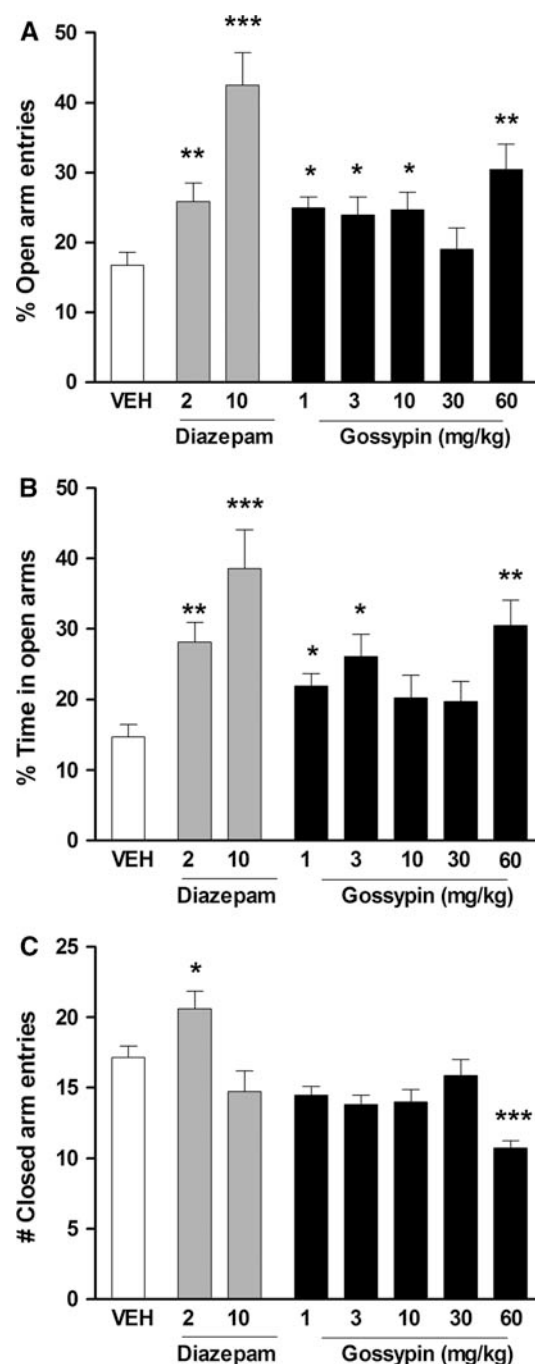
**Fig. 2** Effect of diazepam (2 and 10 mg/kg) and myricitrin (1–30 mg/kg) on the behaviour of mice in the elevated plus maze. Bars represent mean  $\pm$  SEM ( $n = 12$ /group) of the % open arm entries (a), the % time spent on the open arms (b), and number of closed arm entries (c), recorded over a 5 min session, 20 min after an i.p. injection of drugs or vehicle. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test

Figure 5 depicts the effects of flavone on the various parameters of the EPM. Flavone induced a significant increase, when compared to vehicle treatment, in the % of



**Fig. 3** Effect of diazepam (2 and 10 mg/kg) and naringin (1–30 mg/kg) on the behaviour of mice in the elevated plus maze. Bars represent mean  $\pm$  SEM ( $n = 12$ /group) of the % open arm entries (a), the % time spent on the open arms (b), and number of closed arm entries (c), recorded over a 5 min session, 20 min after an i.p. injection of drugs or vehicle. \* $P < 0.05$  or \*\*\* $P < 0.001$  compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test

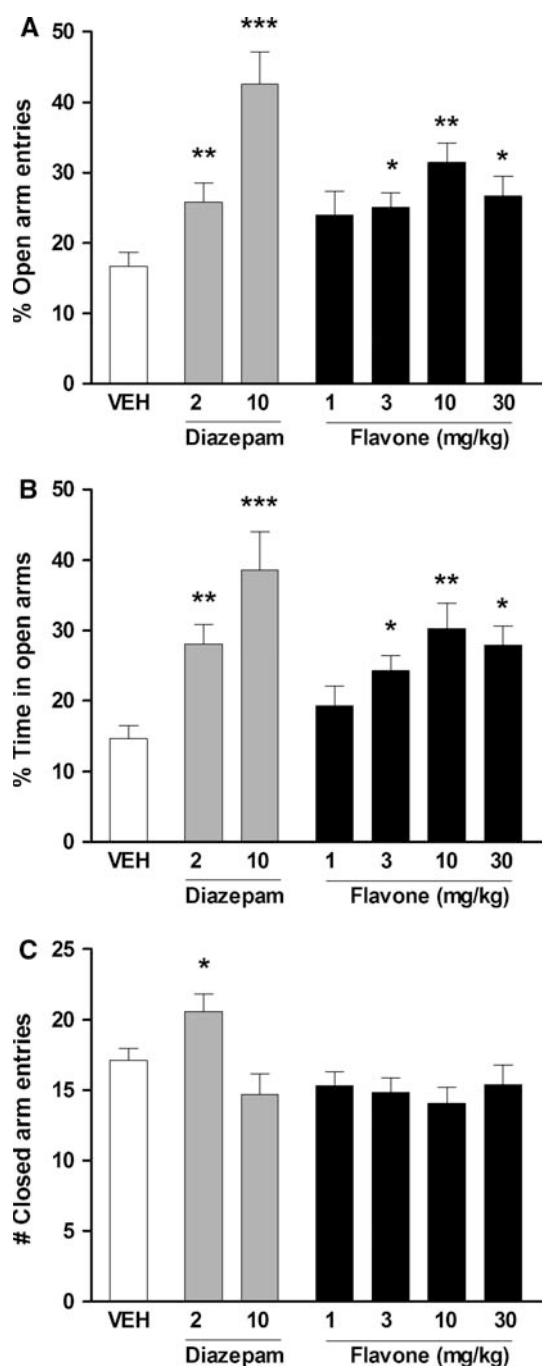
open arm entries and the % of time spent in the open arms, at doses of 3 ( $P < 0.05$ ), 10 ( $P < 0.01$ ) and 30 mg/kg ( $P < 0.05$ , Fig. 5a, b). The influence of treatment on



**Fig. 4** Effect of diazepam (2 and 10 mg/kg) and gossypin (1–60 mg/kg) on the behaviour of mice in the elevated plus maze. Bars represent mean  $\pm$  SEM ( $n = 12$ /group) of the % open arm entries (a), the % time spent on the open arms (b), and number of closed arm entries (c), recorded over a 5 min session, 20 min after an i.p. injection of drugs or vehicle. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test

activity-related parameters like the number of closed entries did not reach statistical significance at any of the doses tested ( $P > 0.05$ , Fig. 5c).





**Fig. 5** Effect of diazepam (2 and 10 mg/kg) and flavone (1–30 mg/kg) on the behaviour of mice in the elevated plus maze. Bars represent mean  $\pm$  SEM ( $n = 12$ /group) of the % open arm entries (a), the % time spent on the open arms (b), and number of closed arm entries (c), recorded over a 5 min session, 20 min after an i.p. injection of drugs or vehicle. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test

#### Measure of Locomotor Activity

Locomotor activity of treated animals was measured immediately after completion of the EPM paradigm. Results from this test are summarized in Table 1.

Diazepam, at a dose of 10 mg/kg, caused a significant reduction in the number of beam breaks revealing a consistent with a reduction in the locomotor activity when compared to the vehicle group ( $P < 0.01$ ). In contrast, the 2 mg/kg dose had no significant effect.

From all the flavonoids tested only naringin and gossypin seemed to have an effect on the locomotor activity at high doses. Naringin 30 mg/kg, the highest dose tested, significantly reduced the number of beam breaks ( $P < 0.01$ , Table 1), while gossypin induced a similar reduction at a dose of 60 mg/kg ( $P < 0.05$ , Table 1). All other doses of these two flavonoids, together with all doses tested of flavone and myricitrin showed no significant difference when compared to vehicle-treated animals.

#### Horizontal Wire Test

The results of the horizontal wire test are summarized in Table 1. Mice treated with diazepam showed a dose-dependent impairment in their performance to grasp the wire. Statistical significance for this effect was reached at doses of 10 and 30 mg/kg ( $P < 0.05$ , Table 1). Flavonoids were tested at the highest dose feasible according to their solubility in the vehicle used. Myricitrin and naringin, at 30 mg/kg, reduced the percentage of mice grasping the wire but this response did not reach statistical significance when compared to vehicle-treated animals. On the other hand, gossypin and flavone were without effect at doses of 60 and 30 mg/kg, respectively.

#### Discussion

Pharmacological treatment is often used as the first-line approach for the management of anxiety-related disorders. Unfortunately, the medications that are currently used have undesirable side effects and a significant number of patients are resistant to these first-line treatments [21, 22]. Consequently, the demand for alternative pharmacological agents is growing. The EPM is a rodent model of anxiety that has been widely used in the search for novel anxiolytic compounds. It is regarded as an 'approach-avoidance' model because it exploits the conflicting tendencies of a rodent to naturally explore novel environments (approach) versus their innate aversion for potentially dangerous open spaces (avoidance) [23]. Consequently, rodents spend the majority of the test session in the closed arms of the maze. This test has been validated for rats and mice, and proven to detect both anxiolytic and anxiogenic effects of pharmacological agents [19]. Anxiolytic drugs increase exploration of the open arms, while anxiogenic drugs reduce open arms activity. As the EPM is based on exploration, this test is dependent on the motor function of the mice.

**Table 1** Effect of diazepam and the flavonoids myricitrin, naringin, gossypin and flavone on the ability of mice to grasp the wire and on the spontaneous locomotor activity

Treatment	Dose (mg/kg, i.p.)	Wire test		Locomotor activity	
		% mice grasping wire	<i>n</i>	Mean $\pm$ SEM of number beam breaks	<i>n</i>
Vehicle	–	100	17	702 $\pm$ 25	25
Diazepam	2	75	12	650 $\pm$ 36	17
	10	43*	14	502 $\pm$ 34 <sup>##</sup>	9
	30	0*	14	ND	–
Myricitrin	1	ND	–	672 $\pm$ 20	10
	3	ND	–	724 $\pm$ 27	11
	10	ND	–	670 $\pm$ 38	10
	30	73	11	672 $\pm$ 32	10
Naringin	1	ND	–	707 $\pm$ 33	11
	3	ND	–	764 $\pm$ 42	13
	10	ND	–	633 $\pm$ 37	12
	30	84	13	452 $\pm$ 75 <sup>##</sup>	12
Gossypin	1	ND	–	687 $\pm$ 31	12
	3	ND	–	653 $\pm$ 22	15
	10	ND	–	620 $\pm$ 27	14
	30	ND	–	624 $\pm$ 36	14
Flavone	60	100	15	560 $\pm$ 43 <sup>#</sup>	12
	1	ND	–	753 $\pm$ 31	15
	3	ND	–	668 $\pm$ 34	15
	10	ND	–	663 $\pm$ 42	13
	30	100	14	770 $\pm$ 32	17

ND not determined

\*  $P < 0.01$  different from vehicle; Chi-square test#  $P < 0.05$ , ##  $P < 0.01$  different from vehicle; Dunn's multiple comparison test after Kruskal–Wallis test

Thus, careful measures of locomotor activity provide support for interpretation of drug effects as being specific to anxiety-related behaviour. In the EPM, locomotor activity is assessed by monitoring either the total arm entries or the number of closed arm entries, the latter being a more accurate measure of motor activity [19]. Hence, the number of closed arm entries was determined in this investigation. Ambulatory locomotor activity was further assessed in the actimeter immediately after mice completed a session in the EPM.

Locomotor activity is in turn dependent on proper control of muscle activity. Muscle relaxation is commonly associated with sedation [20], which may be indicated by less closed arm entries in the EPM and less counts in the actimeter. Myorelaxation may also impair the exploratory activity of the mice and thus affect their performance in the EPM. Hence, the horizontal wire test was carried out to detect if any of the examined flavonoids possessed myorelaxant effects.

In this study, diazepam was used as a positive control because it's a well-known for its anxiolytic, sedative and myorelaxant effects. Diazepam 2 mg/kg significantly increased open arm exploration in the EPM with no significant changes in the locomotor activity indices, compared to the vehicle group. The 2 mg/kg dose caused 25% of treated mice to fail the horizontal wire test, however, this was not considered statistically significant. These results indicate that a 'true' anxiolytic effect was detected in the EPM. Hence, this validates the ability of our EPM to detect anxiolytic agents. Interestingly, diazepam 10 mg/kg caused a much larger increase in the open arm parameters compared to the 2 mg/kg dose. However, this dose also caused a significant reduction in locomotor activity, as detected by the number of closed arm entries and specially the actimeter. Furthermore, 57% of treated mice failed the horizontal wire test. When injected at 30 mg/kg, diazepam impaired the ability of 100% of the animals to grasp the wire, evidencing a significant myorelaxant effect. These results confirmed that the administration of diazepam on animals leads to a series of pharmacological actions within a narrow dose range [18].

From the flavonoids tested, myricitrin and flavone were effective on the EPM showing a clear anxiolytic effect with no signs of sedation. In the case of myricitrin, a dose of 1 mg/kg significantly increased open arm exploration evidencing its anxiolytic action, which surprisingly diminished with increasing doses. Furthermore, 30 mg/kg of myricitrin impaired 27% of animals of grasping the wire and although this effect was not statistically significant, it might reveal a weak myorelaxant action of this drug. Due to solubility issues, higher doses of this compound could not be tested and this hypothesis remains unclear.

In turn, flavone increased open arm exploration in the EPM in a dose dependent manner. Maximal anxiolytic effect was reached at 10 mg/kg. As mentioned before, flavones did not induce significant changes in locomotion or muscle control. These results are in good agreement with those of Marder et al. [24], who also reported that flavone exhibited significant anxiolytic effects in the EPM at doses ranging between 3 and 10 mg/kg. The same study also showed that flavone did not cause sedation, myorelaxation or significant reduction in locomotor activity up to a dose of 10 mg/kg.

Gossypin and naringin both shared a similar profile in our experiments, with low doses inducing a robust anxiolytic effect comparable to that of diazepam 2 mg/kg, which diminished with increasing doses of the flavonoids. Interestingly, high doses of these two flavonoids showed a dramatic increase in the open arm exploration accompanied by a decrease in locomotor activity as evidenced by a reduction in closed arm entries in the EPM, and the number of beam breaks in the actimeter. Hence, naringin and

gossypin seem to induce anxiolytic and sedative effects at high doses. In addition, naringin at 30 mg/kg appeared to have a slight myorelaxant effect in the horizontal wire test, although it was not considered statistically significant, while gossypin was without effect.

The abovementioned effects elicited by these flavonoids are the consequence of interaction with specific biological targets. In vitro data regarding interactions of these agents with different proteins is abundant and information for each flavonoid is ought to be compiled in order to find a possible mechanism of action that could explain these in vivo actions. Flavone, and flavone derivatives, have been extensively studied as positive modulators of GABA<sub>A</sub> receptors, and their in vivo effects have been well correlated with this action [24].

Other relevant targets of flavonoids include ion channels, such as inwardly-rectifying potassium channels (GIRK) and human ether-a-go-go-related gene (hERG) voltage-dependent potassium channels. Indeed, flavone has been shown to inhibit both classes of channels and gossypin, myricitrin and naringin are positive modulators of GIRK channels [25, TY unpublished results], and that might account for their distinct in vivo profile. GIRK channel subunits are expressed in various regions of the central nervous system and they may be involved in various CNS functions such as cognition, memory, emotions and motor coordination [26]. Similarly, ether-a-go-go-related genes (ERGs) are highly expressed in various parts of CNS including the hippocampus, olfactory bulb, paleocortex, limbic structures, and the cerebellum [27]. Hence, the modulation of these potassium channels by flavonoids may play a role in the in vivo effects described in this work.

Flavone is also a moderate to weak negative modulator of  $\alpha 4\beta 2$ -nAChRs and GABA<sub>C</sub> receptors expressed in *Xenopus laevis* oocytes [28]. The study by Chu et al. [28] also showed that naringin is a negative modulator of  $\alpha 4\beta 2$ -nAChRs. The nicotinic-cholinergic systems are thought to modulate anxiety and stress response [29]. On the other hand, gossypin activates opioid receptors, as it exhibits analgesic effects that are antagonized by naloxone [7]. Opioid receptor activation could account for the sedation experienced with gossypin at high doses. Myricitrin also exhibits analgesia, although this is not via opioid receptor activation [8, 30]. The mechanism behind its antinociceptive action is thought to involve Gi/o protein activation, opening of voltage-gated and small-conductance- calcium-gated potassium channels, and inhibition of calcium influx. This evidence strongly suggests that a variety of receptor systems are responsible for the behavioural responses detected in our study.

Another important factor to be considered is the bioavailability and metabolism issues surrounding the flavonoids, especially glycosides. It is generally accepted that flavonoids are absorbed as aglycones, since flavonoid

glycosides are fairly large and highly polar compounds that do not readily cross membranes [31, 32]. Furthermore, flavonoids undergo extensive metabolism in the liver and kidneys, but also in other tissues. These compounds are good substrates and inducers of phase II enzymes [31] and the resulting biotransformation may lead to reduced biological activity or the production of active metabolites. The flavonoid glycosides in particular undergo hydrolysis by glycosidases present in the cells of the gastrointestinal mucosa or secreted by colonic bacteria [33, 34]. This is a major issue, as the sugar moiety of the flavonoid glycosides is required for the CNS depressant effects seen for hesperidin, naringin and gossypin [6]. In this study, flavonoids were administered by intraperitoneal injection to minimise these metabolism issues. However, hydrolysis of the sugar moiety may still occur since many other tissues express hydrolase enzymatic activity. For example, lactase phlorizin hydrolase is present in the brush border of the small intestine. Hence, hydrolysis of certain flavonoid glycosides can occur within the gut lumen [35]. Bioavailability and distribution of flavonoids glycosides is also extremely complex and poorly understood. For example, Tsai and collaborators [36] showed that following an intravenous administration of naringin at 30 mg/kg, this flavonoid is not detected in the brain dialysate of rats, but was found when injected at 100 mg/kg. In contrast, both naringenin and its glucuronide were detected in the cerebral cortex following intravenous administration of naringenin 20 mg/kg [37]. Altogether, it seems that defining the exact mechanism of action of flavonoid glycosides on the CNS requires a multidisciplinary approach designed to define active metabolites, brain concentrations and interaction with target proteins involved in the regulation of anxiety.

In conclusion, this is the first study documenting the anxiolytic action of the flavonoid glycosides gossypin, myricitrin and naringin. Our results and the ones of others suggest that these agents have the potential to exert range of biological activities that also include sedation, myorelaxation and analgesia.

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