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Route of administration determines the anxiolytic activity of the flavonols kaempferol, quercetin and myricetin — are they prodrugs?

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Abstract

Several *in vivo* and *in vitro* studies have confirmed that flavonols are metabolized by the intestinal microflora to their corresponding hydroxyphenylacetic acids. In this article, a comparison of the anxiolytic activity of the flavonols kaempferol, quercetin and myricetin in the elevated plus maze after oral (po) and intraperitoneal (ip) administration to mice in a dose range of 0.1 to 2.0 mg/kg is presented. In addition, their corresponding metabolites *p*-hydroxyphenylacetic acid (*p*-HPAA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were tested after intraperitoneal administration. Anxiolytic activity was detected for kaempferol and quercetin only after oral administration. No anxiolytic effects were observed when kaempferol and quercetin were given via the intraperitoneal administration route. The corresponding hydroxyphenylacetic metabolites *p*-HPAA and DOPAC showed anxiolytic effects after intraperitoneal application. In order to further test the hypothesis that flavonoids are possible prodrugs which require activation by intestinal bacteria, gut sterilization was performed using pretreatment with the antibiotic enrofloxacin (7.5 mg/day, po, for 4 days). After antibiotic treatment, the anxiolytic effect of kaempferol and quercetin disappeared, whereas it was still present for the positive control diazepam. Our results support the hypothesis that flavonoids act as prodrugs which are transformed into their active hydroxyphenylacetic acid metabolites by intestinal microflora.

Keywords: Flavonoids; Prodrugs; Intestinal microflora; Gut sterilization; *p*-Hydroxyphenylacetic acid; DOPAC

1. Introduction

Recent clinical trials demonstrated that there is a clear correlation between dietary patterns and depression, suggesting that a diet rich in fruits and vegetables is associated with a decreased prevalence of depression, whereas high consumption of processed food, such as meat or fried food, presents a clear risk factor for depression [1,2]. A common feature of fruits and vegetables is that they are the major dietary sources of flavonoids. The beneficial health effects of flavonoids have been related to their antioxidant, anti-inflammatory, antiestrogenic, cardioprotective, cancer chemopreventive, neuroprotective, antidepressant and anxiolytic effects [3-9]. In particular, the flavonols quercetin and kaempferol exhibited antidepressant effects in several studies. It has been demonstrated that both compounds act as monoamine oxidase inhibitors [10,11]. Recent findings from our group suggest that the route of administration determines whether flavonols such as kaempferol exert *in vivo* anxiolytic effects in the elevated plus maze in mice. After oral application, kaempferol showed significant anxiolytic effects, whereas no behavioral changes were detected following intraperitoneal administration in mice [12].

Although it is well known that flavonoids undergo transformation by colonic microorganisms into small phenolic acids, such as *p*-hydroxyphenylacetic acid, phenylacetic acid, 3-phenylpropionic acid, *m*-hydroxyphenylacetic acid, *p*-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid and others [13-16], little is known about the fate of these metabolites, as well as their physiological effects.

The flavonols kaempferol, quercetin and myricetin (Fig. 1), which were used as model compounds in the present study, merely differ in the number of hydroxyl groups at the B-ring. These compounds share equal degradation pathways. After cleavage of the C-ring they are converted to *para* hydroxyphenylacetic acid (*p*-HPAA), 3,4-dihydroxyphenylacetic acid (DOPAC) with subsequent degradation to *meta*-hydroxyphenylacetic acid (*m*-HPAA) and 3,4,5-trihydroxyphenylacetic acid with subsequent degradation to 3,5-dihydroxyphenylacetic acid, respectively [17-19] (Fig. 1). However, as mentioned above, the biological properties of these microbial metabolites have rarely been examined.

The objective of this research was therefore to investigate the hypothesis that flavonoids are precursors of active metabolites (i.e., are prodrugs). The elevated plus maze in mice was used to evaluate the anxiolytic effects of the flavonols kaempferol, quercetin and myricetin and their corresponding metabolites *p*-HPAA and DOPAC after oral and intraperitoneal administration. To further investigate the putative effect of the intestinal flora on the bioactivity of kaempferol, quercetin

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and myricetin, gut sterilization via oral administration of the antibiotic enrofloxacin was performed prior to oral application of the test compounds.

2. Material and methods

2.1. Chemicals

3,4-Dihydroxyphenylacetic acid (DOPAC), *p*-hydroxyphenylacetic acid (*p*-HPAA), quercetin (purity 98%) and enrofloxacin were purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA); kaempferol (purity 98%) from Chromadex, Inc. (Irvine, CA, USA); and myricetin (purity 98%) from Extrasynthèse S.A.S. (Genay, France). Diazepam (10 mg/5 ml; Hospira, Inc., Lake Forrest, IL, USA) was diluted to 1.0 or 1.5 mg/10 ml with deionized water (Millipore quality) containing 0.5% propylene glycol.

Four different doses (0.1, 0.5, 1.0 and 2.0 mg/kg) of kaempferol, quercetin and myricetin were suspended in 10 ml deionized water with 0.5% propylene glycol. 3,4 Dihydroxyphenylacetic acid (DOPAC) and *p*-hydroxyphenylacetic acid (*p*-HPAA) were tested in three different doses (0.1, 0.5 and 1.0 mg/kg) and were prepared with isotonic saline. All compounds were administered in a volume of 0.1 ml/10 g body weight of mice. Enrofloxacin was used in a dose of 7.5 mg/kg diluted in deionized water and administered orally for four consecutive days. All solutions were prepared freshly on test days.

2.2. Animals

Male C57BL/6 mice at the age of 6 weeks weighing 20–25 g were purchased from Harlan (Indianapolis, IN, USA). Mice were housed in cages of five at 20±1°C in a 12-h light/dark cycle. Tap water and standard food pellets (Harlan Teklad, Indianapolis, IN, USA; standard diet) were available *ad libitum*. The mice were randomly assigned to different treatment groups of 12 animals and tested in a varying order. The animals were tested repeatedly under the same experimental conditions between 8:00 a.m. and 1:00 p.m. All animals were housed and all experiments were performed according to the policies and guidelines of the International Animal Care and Use Committee (IACUC) of the University of Florida, Gainesville, FL, USA (protocol no. 200903386).

2.3. Drug administration

The animals were assigned randomly to different treatment groups (n=12). For the oral administration, the experimental animals were treated with diazepam (1.0 mg/kg) as positive control or one of the flavonoids kaempferol, quercetin or myricetin (0.1, 0.5, 1.0 and 2.0 mg/kg). Control animals received vehicle (0.5% propylene glycol in deionized water) in an equal volume according to their body weight. The intraperitoneal administration was performed using diazepam (1.5 mg/kg) as positive control and one of the flavonoid metabolites *p*-HPAA or DOAPC (0.1, 0.5 and 1.0 mg/kg) as well as kaempferol and quercetin (0.5 and 1.0 mg/kg). Control animals received vehicle (0.5% propylene glycol in 0.9% sodium chloride solution). All animals were treated 60 min before evaluation in the maze.

2.4. Gut sterilization

To determine the role of the bacterial microflora in the intestinal metabolism of flavonoids, it was necessary to inactivate the bacterial microflora performing gut sterilization. Based on the study of Johnson et al. [20], each individual was treated orally with the antibiotic enrofloxacin (7.5 mg/kg) for four consecutive days. The last antibiotic dose was administered the day before the behavioral experiment was performed. Completion of gut sterilization was performed according to the study of Johnson et al. [20].

2.5. Elevated plus maze

Anxiolytic activity was measured using the elevated plus maze test (EPM) based on our previously published data [5,12,21]. Briefly, the mice were individually placed on the center of the maze facing a closed arm. The number of entries and the time spent on the open and closed arms were recorded during a 6-min observation period. An arm entry was defined as entry of all four paws into an arm. Animals with no entries to an open arm were eliminated from analysis. The percentage time spent on open arms (100×open/total time) was calculated for each animal. The distance covered by each animal was measured, representing locomotor activity. Each experiment was videotaped using a high-resolution video camera. The video analysis was performed

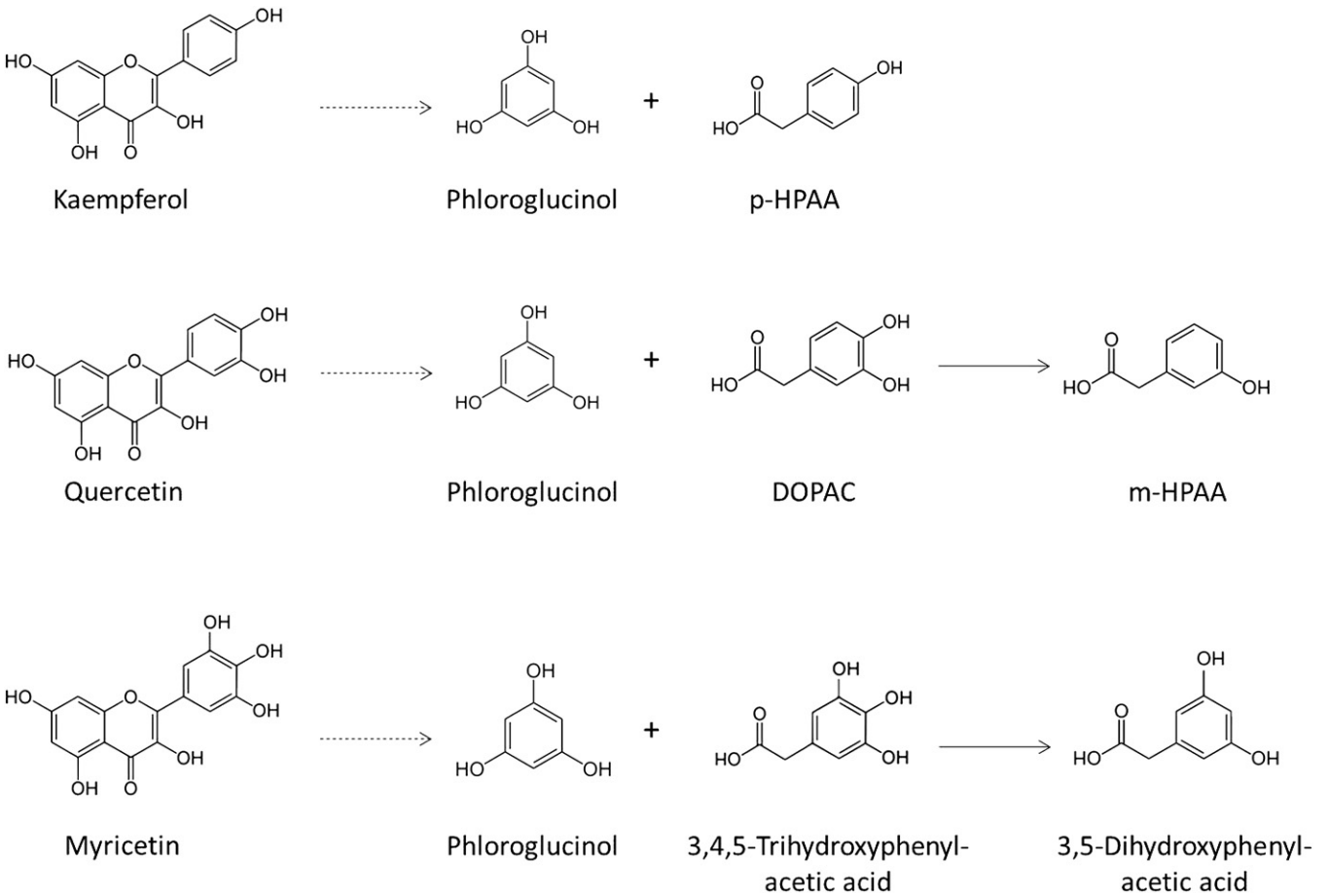

Fig. 1. Degradation pathway of the flavonols kaempferol, quercetin and myricetin. DOPAC, 3,4 Dihydroxyphenylacetic acid; *m*-HPAA, *meta*-hydroxyphenylacetic acid; *p*-HPAA, *para*-hydroxyphenylacetic acid. Note: The proposed pathway displays only a part of a more complex degradation pathway as more metabolites can be detected [17].

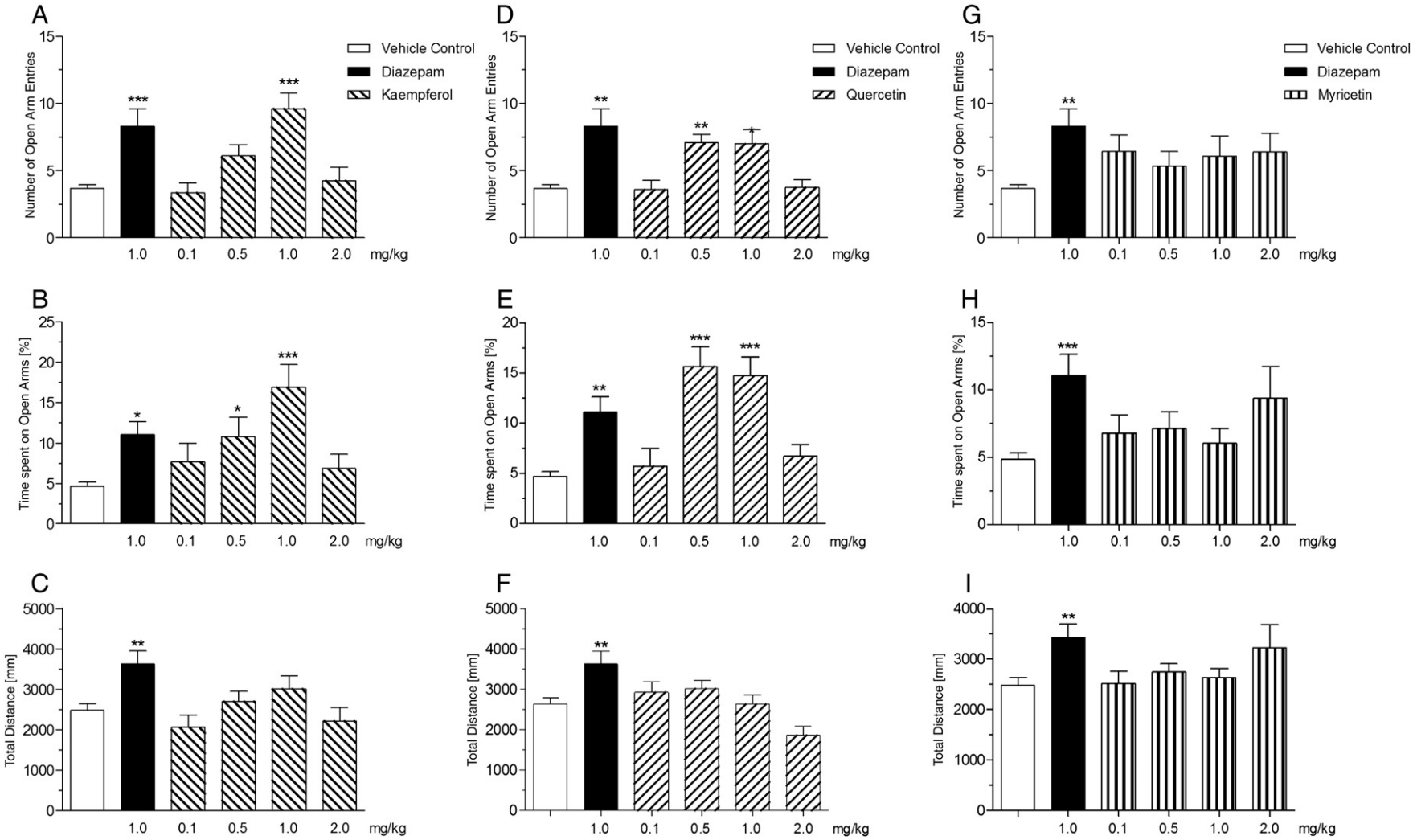

Fig. 2. Anxiolytic effects of kaempferol, quercetin and myricetin (0.1, 0.5, 1.0 and 2.0 mg/kg) after oral administration illustrated by the number of open-arm entries (A, D, G), the percentage time spent in open arms (B, E, H) and the total distance travelled in the elevated plus maze in 6 min (C, F, I). Results are expressed as mean±S.E.M.; *P<.05 vs. control; **P<.01 vs. control; ***P<.001 vs. control, ANOVA with Dunnett's multiple comparison test.

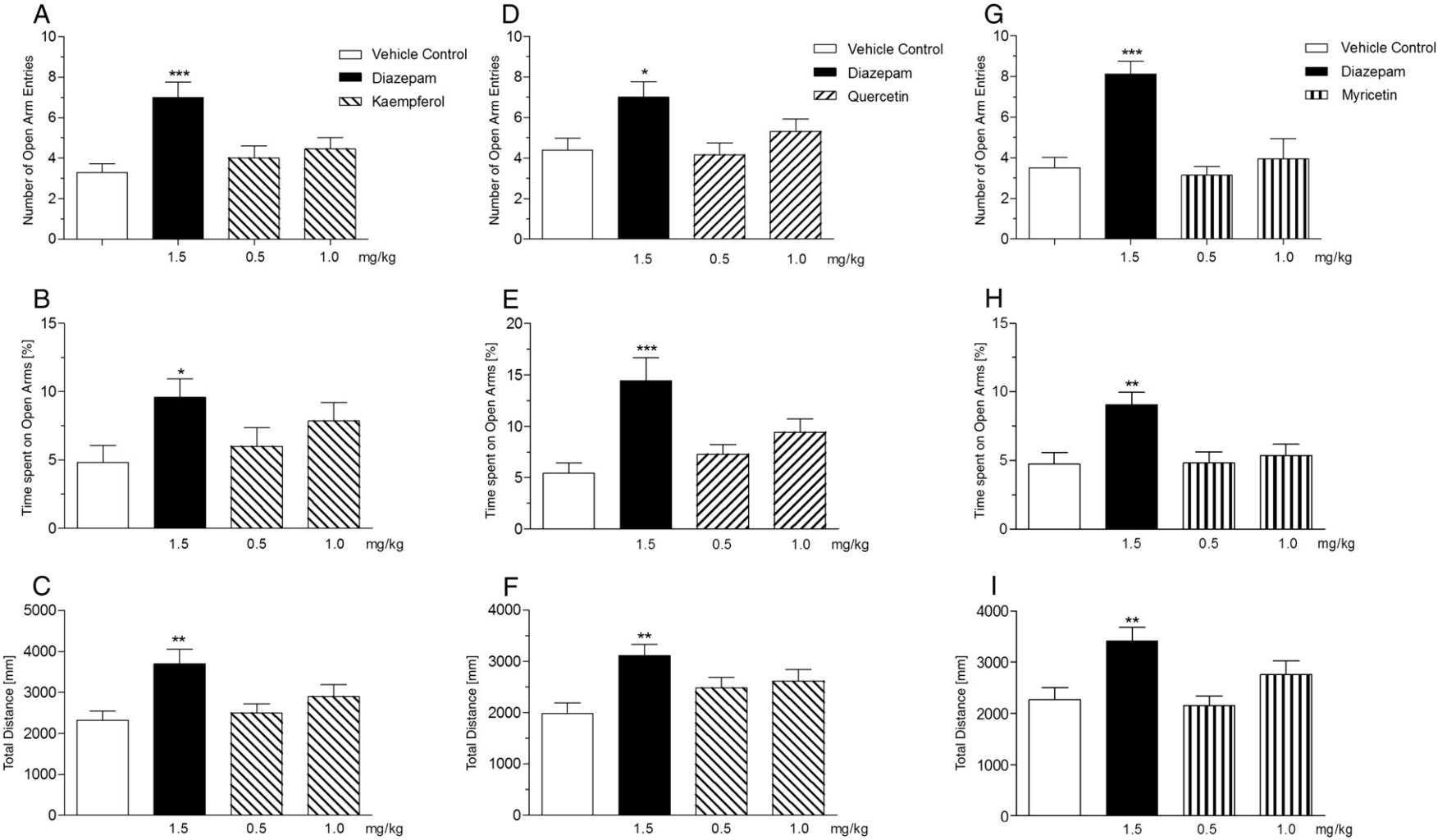

Fig. 3. Anxiolytic effects of kaempferol, quercetin and myricetin (0.1, 0.5, 1.0 and 2.0 mg/kg) after intraperitoneal injection illustrated by the number of open-arm entries (A, D, G), the percentage time spent in open arms (B, E, H) and the total distance travelled in the elevated plus maze in 6 min (C, F, I). Results are expressed as mean±S.E.M.; *P<.05 vs. control; **P<.01 vs. control; ***P<.001 vs. control, ANOVA with Dunnett's multiple comparison test.

using TopScan, Top View Animal Behavior Analyzing System (version 1.00, Clever Sys, Inc., Preston, VA, USA).

2.6. Statistical analysis

The calculation of the percentage time and number of entries on the open arms with 95% confidence limits and comparisons of the results were performed using GraphPad Prism (version 5.00, GraphPad Software, Inc., San Diego, CA, USA). The statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test.

3. Results

3.1. Anxiolytic activity of the flavonoids kaempferol, quercetin and myricetin after oral and intraperitoneal administration

One-way analysis of variance revealed a significant increase in number of open-arm entries after administration of the positive control diazepam as compared to control (Fig. 2A and D). The percent time spent on open arms was also significantly increased for diazepam if compared to the control group (Fig. 2B and E). Oral administration of kaempferol and quercetin induced an increase of open-arm entries in 0.5 and 1.0 mg/kg (Fig. 2A and D). Similar to the numbers of open-arm entries, the time spent in the open arms was significantly increased after oral administration of kaempferol and quercetin (Fig. 2B and E). Interestingly, the anxiolytic effects disappeared when both compounds were administered in a dose of 2.0 mg/kg, indicating a U-shaped activity. Oral administration of myricetin in a dose range from 0.1 to 2.0 mg/kg did not cause any significant changes in elevated plus maze parameters (Fig. 2G and H). None of the flavonoid compounds showed any significant differences in the total distance traveled on the EPM (Fig. 2C, F and I). However, a significant increase in the total distance traveled was observed for diazepam. Interestingly, when kaempferol and quercetin were administered intraperitoneally in the same doses where they exerted anxiolytic effects after oral dosing (0.5 and 1.0 mg/kg), neither changes in the total number of open-arm entries nor differences in the time spent on open arms were observed (Fig. 3A, B, D, E, G and H).

3.2. Influence of the hydroxylation pattern in ring B on anxiolytic activity

In order to determine whether the B-ring hydroxylation pattern of flavonols affects the anxiolytic activity of kaempferol, quercetin and myricetin, the number of open-arm entries as well as the time spent on open arms was compared for the oral dose of 1 mg/kg. Linking the number of the open-arm entries after oral administration of kaempferol, quercetin and myricetin, it was found that the anxiolytic activity decreased with an increasing number of hydroxyl groups in ring B (Fig. 4A). Kaempferol with one hydroxyl group in ring B exhibited the strongest anxiolytic activity. The same tendency was observed for the percentage time spent in the open arms. The anxiolytic activity represented by the percentage time spent in open arms was decreasing with an increasing number of hydroxyl groups (Fig. 4B).

3.3. Anxiolytic activity of the main metabolites p-HPAA and DOPAC after intraperitoneal administration

Intraperitoneal administration of the kaempferol metabolite *p*-HPAA exhibited a significant anxiolytic effect expressed by a significant increase of open-arm entries and percentage time spent on open arms at 0.5 and 1.0 mg/kg (Fig. 5A and B). Interestingly, the lower dose of 0.5 mg/kg was more active than 1.0 mg/kg. For the quercetin metabolite DOPAC, a significant effect was observed for the percentage time spent on open arms in the dose of 1.0 mg/kg, whereas only a trend for increased number of open-arm entries was

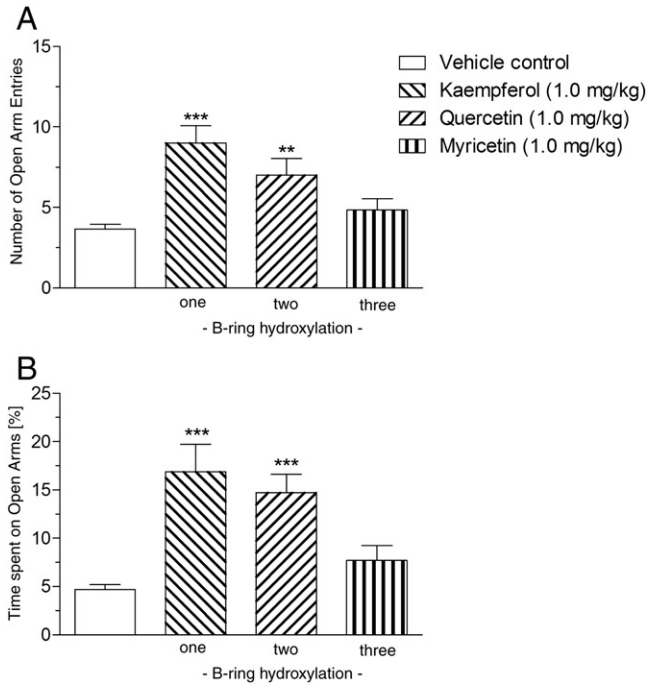

Fig. 4. Influence on the anxiolytic activity of the hydroxylation pattern in ring B after oral administration of kaempferol, quercetin and myricetin (1.0 mg/kg). (A) Number of open-arm entries; (B) percentage time spent in open arms in the elevated plus maze. Results are expressed as mean±S.E.M.; $^{**}P<.01$ vs. control; $^{***}P<.001$ vs. control, ANOVA with Dunnett's multiple comparison test.

detected (Fig. 5D and E). Neither *p*-HPAA nor DOPAC affected locomotor activity, whereas diazepam significantly increased the total distance travelled in 6 min (Fig. 5C and F).

3.4. Anxiolytic activity of orally administered kaempferol and quercetin after antibiotic treatment with enrofloxacin

In order to assess the role of colonic microflora for the potential bioactivity of kaempferol, quercetin and myricetin, gut sterilization via oral enrofloxacin treatment for four consecutive days prior to oral treatment was performed. As shown in Fig. 6, gut sterilization led to a loss of anxiolytic activity for kaempferol and quercetin after oral treatment (Fig. 6A, B, D and E). However, although lightly diminished, diazepam in a dose of 1.0 mg/kg still significantly increased both *open-arm* time and *% open-arm entries* in the EPM.

4. Discussion

It has been suggested that flavonoids may act to protect cells by more complex mechanisms than was once thought [22]. It has also become clear that the bioactive forms of flavonoids *in vivo* are not those forms found in plants. It is further well known that flavonoids are poorly absorbed from the gastrointestinal tract and that colonic bacteria can convert flavonoids into simple phenolic acids [23]. However, little is known about the extent of absorption and, more importantly, about the potential health-promoting effects of phenolic acids. Konishi [24] showed that *p*-HPAA and DOPAC can be absorbed from the gut lumen via an active transporter, the monocarboxylic acid transporter (MCT), as well as via passive diffusion and attain in this way the systemic circulation. Interestingly, MCTs are also expressed on the luminal membrane of endothelial cells at the blood–brain barrier [25]. Their primary role is in the transport of monocarboxylate solutes, such as lactate and pyruvate, into the brain. Thus, it seems likely that phenolic acids could be transported to the

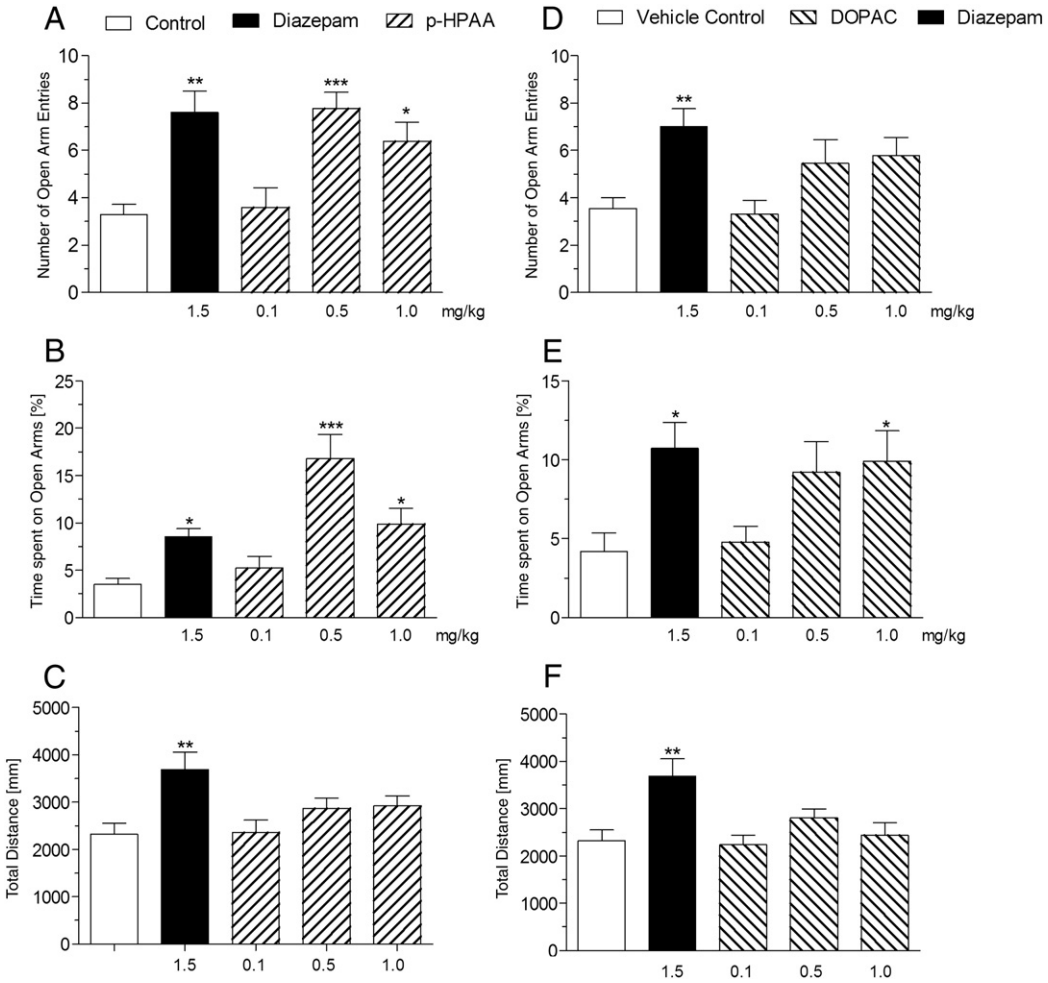

Fig. 5. Anxiolytic effects of *p*-HPAA (A–C) and DOPAC (D–F) after intraperitoneal administration expressed by the number of open-arm entries, the percentage time spent in open arms and the total distance travelled in the elevated plus maze. Results are expressed as mean±S.E.M.; *$P<.5$ vs. control; **$P<.01$ vs. control; ***$P<.001$ vs. control, ANOVA with Dunnett's multiple comparison test.

brain where they act as modulators of brain function and, in particular, exert antidepressant and/or antianxiety effects.

This present article represents one of the first studies investigating the hypothesis that flavonoids are precursors of active metabolites (i.e., are prodrugs). In general, a prodrug must undergo metabolic conversion before becoming an active pharmacological agent. The possibility that flavonoids could be prodrugs was first discussed by Kim et al. [26] who demonstrated *in vitro* that the intestinal bacteria metabolites of rutin and quercetin, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid, respectively, possess more effective antiplatelet activity than the parent compounds. Two recent studies showed that flavonoid metabolites from colonic microbiota exert anti-inflammatory [27] and anti-apoptotic activities [28] *in vivo*.

In our present study, kaempferol and quercetin showed a significant anxiolytic effect in the EPM by increasing both the total number of open-arm entries and the percentage time spent on the open arms at the concentrations of 0.5 and 1.0 mg/kg after oral administration. In a concentration of 2 mg/kg, the anxiolytic activity decreased, indicating a U-shaped activity. It can be speculated that this concentration causes a leakage of the tight junctions in the intestine so that the flavonoids are absorbed without further metabolization, therefore decreasing the activity. Following intraperitoneal injection, neither compound showed any anxiolytic effect. The lack of anxiolytic activity after intraperitoneal administration confirms prior findings [29], while it also supports the hypothesis of possible bioactivation in the colon either by the microbial flora or the enterocytes. Although it could be a concern that for both routes of administration the compounds were administered 60 min prior to the behavioral test, this experimental setup was chosen in order to keep the number of possible variables which could influence the overall behavioral outcome relatively small. Furthermore, since the positive control diazepam showed anxiolytic effects 60 min after per os or intraperitoneal administration, it can be assumed that the testing time point is reasonable.

As mentioned above, the flavonols kaempferol, quercetin and myricetin differ from each other with an increasing number of hydroxyl groups in ring B. Comparing the anxiolytic effect of these substances, it was detected that the activity decreases with an increasing number of hydroxyl groups. Kaempferol with one hydroxyl group in ring B revealed the strongest effect, whereas myricetin with three hydroxyl groups exhibited no anxiolytic effect. It can be speculated that the decreased hydrophobic properties as well as steric hindrance could be possible reasons for the missing central effects. The present results provide the first indications that the hydroxylation pattern in ring B seems to have an influence on the intensity of central effects induced by flavonoids; however, further investigations on this subject are necessary.

Based on the previously mentioned hypothesis of bioactivation of kaempferol by intestinal microflora to *para*-hydroxyphenylacetic acid (*p*-HPAA), it was of interest to study the possible anxiolytic effects of

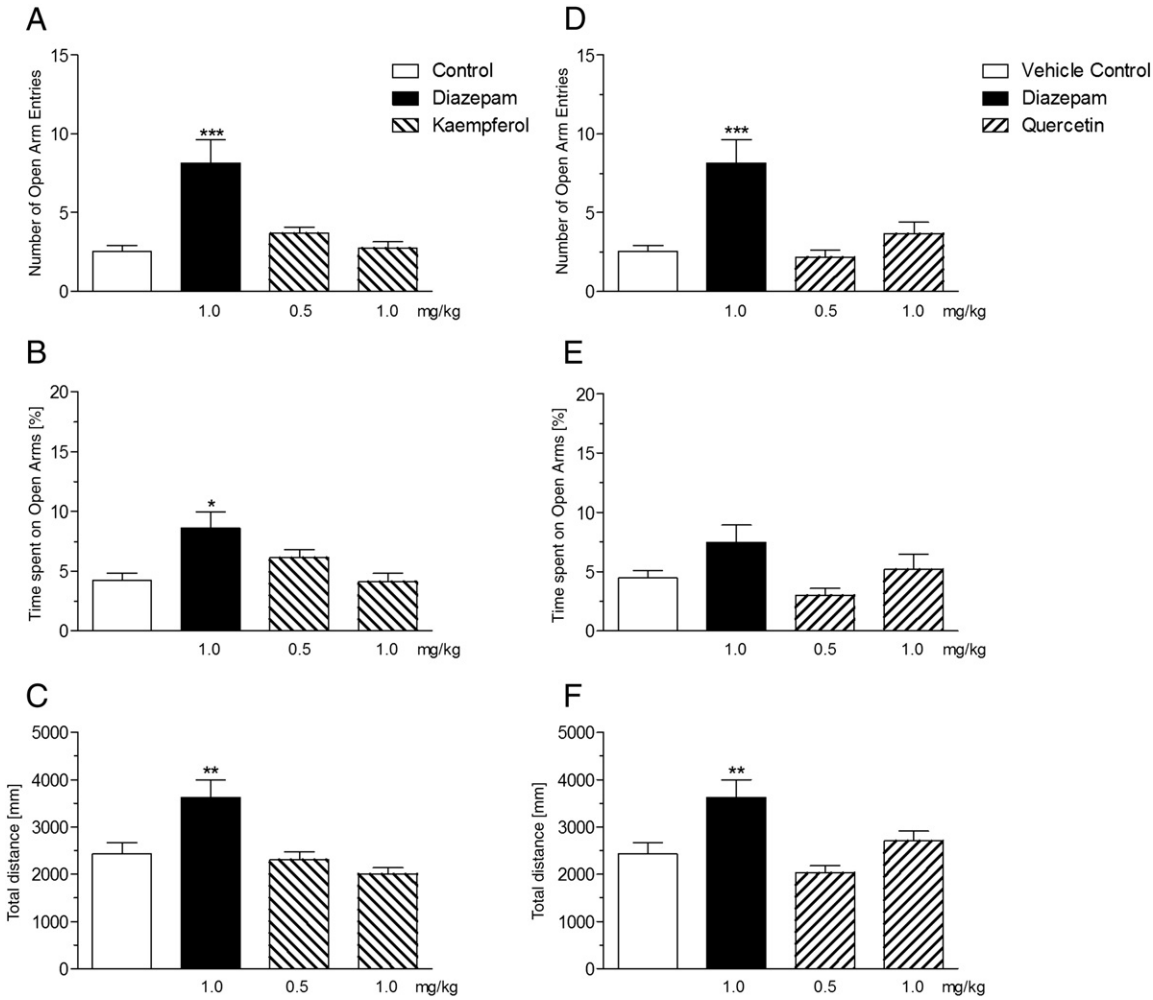

Fig. 6. Anxiolytic effects of oral administration of kaempferol and quercetin (both administered per os) after pretreatment with enrofloxacin (7.5 mg/kg per day, 4 days). Number of open-arm entries (A, D); percentage time spent in open arms (B, E); total distance travelled (C, F) in the elevated plus maze. Results are expressed as mean±S.E.M.; *P<.5 vs. control; **P<.01 vs. control; ***P<.001 vs. control, ANOVA with Dunnett's multiple comparison test.

these metabolites using the EPM in mice. Following the intraperitoneal injection of *p*-HPAA, an anxiolytic effect in the EPM was observed for 0.5 and 1.0 mg/kg. For both compounds, the intraperitoneal route of administration was chosen because it has been shown previously that *p*-HPAA is nitrated, both in the oral cavity and in the stomach, to 3-nitro-4-hydroxyphenylacetic acid, which is excreted through the urine [30]. The doses of 0.5 and 1.0 mg have been chosen hypothetically assuming a complete transformation of kaempferol to *p*-HPAA (e.g., 1 mg kaempferol would be transformed into approximately 0.5 mg *p*-HPAA, assuming no further water content in the compound).

After pretreatment with the antibiotic enrofloxacin for 4 days, diazepam exhibited a significant anxiolytic effect in the number of open-arm entries, whereas no effect was expressed by orally administered kaempferol and quercetin in doses of 0.5 and 1.0 mg/kg. These data further support the hypothesis that the consequential lack of intestinal metabolism obviously prevents kaempferol and quercetin from transforming into their active metabolites. The role of HPAA as a possible biomarker of depression has been discussed in previous studies [31]. It has been shown that during depressive or anxious episodes HPAA concentrations decrease significantly. On recovery, the concentration rises again to its normal level [32]. Interestingly, earlier studies also suggested that most HPAA derives from undigested food residues and that differences in HPAA excretion depend to a considerable extent on differences between individuals in fecal flora [33,34]. Treatment with antibiotic medications resulted in very significant reductions in urinary HPAA excretion, evidence that it is indeed influenced by the condition of the gut flora [35,36].

Interestingly, the metabolites *p*-HPAA, DOPAC and *m*-HPAA occur as decomposition products in the metabolism of trace amines. Trace amines (TAs) are precursors or degradation products of neuroactive biogenic amines in the brain [37]. Their dysregulation has been linked to various psychiatric diseases; for example, significantly low urinary *p*-HPAA concentrations in depressed patients have been reported by several groups [31,35,38,39]. Low *p*-HPAA levels also have been reported in both plasma and cerebrospinal fluid (CSF) of depressed patients [40-43]. Remarkably, treatment with the monoamine oxidase inhibitor maprotiline produced an increase in urinary *p*-HPAA excretion which coincided with clinical improvement [39]. Juorio and McQuade [44] reported increased concentrations of *m*-HPAA and *p*-HPAA in the mouse caudate nucleus after treatment with antipsychotic agents including haloperidol, sulpiride and chlorpromazine. However, it still remains unknown what role the metabolites of the TAs play in the neuronal system, although newer evidence suggests that they might be important for system homeostasis [45]. There are various ways to exert anxiolytic effects in the brain; one way could be an interaction with GABAA receptors mimicking benzodiazepine effects. We recently published data on

the anxiolytic effect of kaempferol and could show that its anxiolytic action is sensitive to flumazenil, a benzodiazepine receptor antagonist at the GABAA site [12]. Experiments supporting this hypothesis are currently under further investigation.

In conclusion, our data provide the first evidence that the route of administration determines the biological activity of flavonoids. The lack of anxiolytic effect of kaempferol and quercetin after intraperitoneal injection as well as gut sterilization with enrofloxacin supports this hypothesis. In addition, we could show for the first time that phenylacetic acids such as *p*-HPAA and DOPAC exert anxiolytic effects *in vivo* which provides further evidence for our proposed hypothesis that flavonoid metabolites are modulators of brain function. However, our present investigation focused only on the anxiolytic activity of flavonoid metabolites, but a much wider activity can be assumed. Larrosa et al. [27], for example, demonstrated the anti-inflammatory activities of flavonoid metabolites, supporting our hypothesis that flavonoid metabolites exert pharmacological activities. More in-depth studies with a wider range of activities are clearly necessary to support our hypothesis. Since flavonoids seem to be prodrugs which undergo metabolic activation before becoming physiologically active, it will be necessary to determine the plasma and tissue levels of phenylacetic acids after oral flavonoid administration and the mechanisms of how they reach the CNS. Further investigations in this direction including uptake, tissue distribution and elimination are currently in progress.

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