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Reactivity of flavonoids with 1-hydroxyethyl radical: a γ-radiolysis study

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Abstract

We have investigated the reactivity between 11 flavonoids and 1-hydroxyethyl radical (HER). HER was recently implicated in many liver injuries induced by ethanol intoxication. In this study, HER was generated by radiolysis; due to its reaction rate, HER is well known to be responsible for solute degradation in irradiated ethanol. Flavonoid ethanol solutions were irradiated with γ-rays and the flavonoid degradation was followed by HLPC. We observed the degradation of flavonols while all other flavonoids (flavones, flavanones, dihydroflavonols, catechins) were not degraded after irradiation. The major radiolysis products were identified by NMR and LC-MS and we concluded that flavonols were essentially transformed into depsides. We proposed a reactivity mechanism between flavonols and HER. In a first step, H-transfer occurred from the 3-OH group to HER. Afterwards, C-ring opening occurred due to the presence of the 2,3-double bond in flavonols. Finally, we calculated the reaction constants in order to evaluate the antioxidant activity of flavonols against HER and to compare it with reference compounds.

Keywords: Flavonoid; 1-Hydroxyethyl radical (HER); γ-Radiolysis; Free radical scavenging

1. Introduction

Ethanol intake has been known to play a causative role in the liver injury for a long time. Di Luzio [1] demonstrated that antioxidant balance of rat hepatic cells was affected by ethanol. Chronic administration of ethanol also caused changes in the structure and function of liver mitochondria [2]. Ethanol was shown to be oxidized to a free radical metabolite, 1-hydroxyethyl radical (HER = $CH_3^{\bullet}CHOH$ also called the α-hydroxyethyl radical), by rat liver microsomal systems and also in vivo in rats and deer mice [3–6]. The radicals were detected by electron paramagnetic resonance (EPR) using spin traps such as 4-pyridyl-1-oxide-*t*-butyl nitrone (POBN) and phenyl-*N*-*tert*-butylnitrone (PBN) [6–9]. HER is implicated in mitochondrial swelling and mitochondrial permeability transition [10]. The ethanol-induced oxidative stress is not restricted to the liver, where ethanol is actively oxidized, but can affect various extrahepatic tissues [11]. The mechanism of HER formation from ethanol has proven to be complex, and two pathways have been proposed [7]. The first one was a $^{\bullet}OH$-dependent reaction and the second one was the direct one-electron oxidation of ethanol by cytochrome P450. Inactivation of physiological antioxidant systems was also reported [12].

The flavonoid family including flavanones, dihydroflavonols, flavones, flavonols, isoflavones, catechins was identified as a major source of antioxidants in fruits, vegetables and beverages. Many epidemiological studies demonstrated correlations between the dietary content in flavonoids and various pathologies including cardiovascular diseases, cancer, and Alzheimer's disease (for a review see Ref. [13]). Numerous in vitro studies also showed that flavonoids possess good anti-inflammatory [14,15], antiviral [16] and enzyme activities [17–19]. Biological activities of flavonoids are attributed in part to their free radical scavenging capacity, especially against hydroxyl and superoxide radicals [19–22]. To our knowledge, little is known concerning antioxidant activity of flavonoids against HER.

The aim of the present work was to study the reactivity of flavonoids toward ethanol-derived radicals. For this, 11

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molecules (including flavanones, dihydroflavonols, flavones, flavonols and catechin) were radiolyzed in ethanol solutions and their degradation was followed by HPLC. Major radiolysis products were identified by NMR and ES-MS. The reactivity mechanism between flavonols and HER was proposed and the reaction constants were calculated and used as an indicator of the efficient HER scavenging capacity of flavonoids.

2. Materials and methods

2.1. Chemicals and reagents

HPLC ethanol and methanol grade (99.8%) were purchased from SdS (Peypin, France), acetic acid from Merck (Darmstadt, Germany). Quercetin, kaempferol, morin, galangin, luteolin, apigenin, chrysin, (±)-taxifolin, (±)-naringenin, (±)-eriodictyol, and (−)-epicatechin were purchased from Sigma (St. Louis, MO, USA) at the highest grade available and purified in our laboratory (>99%).

2.2. Irradiation

Flavonoids were dissolved in ethanol (5×10^{-5}, 10^{-4}, 2.5×10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3} M), and were irradiated in aliquots of 1 ml with doses ranging from 0.2 to 20 kGy at a dose rate of 0.22 Gy/s in a ^{60}Co source carrier type Oris experimental irradiator.

2.3. HPLC analyses

Twenty microliters of flavonoid solutions before and after irradiation was injected into the analytical HPLC system, a Waters model equipped with a 600 model pump, a variable wavelength photodiode array detector (PDA 996) and a 600 model controller. The column used was a 250 × 4.6 mm i.d., 10 μm, μBondapak C18 cartridge (Waters). The mobile phase consisted of 100% methanol (A) and 1% aqueous acetic acid (B). Analyses were performed using a linear gradient from 20% A to 80% A (for galangin, 30% A to 90% A) during 40 min at 1 ml/min.

2.4. Purification

Purification was performed by semi-preparative HPLC using a 100 × 25 mm i.d., 10 μm, μBondapak C18 cartridge (Waters) and the same solvent system as above. The gradient was 20% A to 80% A (for galangin, 30% A to 90% A) at 5 ml/min during 60 min.

2.5. Identification of radiolysis products

The identification of the major radiolysis products of quercetin, morin, kaempferol and galangin was achieved by NMR and LC-MS.

^{1}H-NMR and ^{13}C-NMR spectra were measured in CD_3OD at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR on a Bruker DPX Avance Spectrometer using tetramethylsilane as an internal standard. Complete proton and carbon assignments were based on 1D (^{1}H standard, ^{13}C *J*mod), 2D (^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC) and ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) NMR experiments.

Mass spectroscopy was performed on a Waters Alliance system equipped with a Waters electrospray interface. The source was operated in the negative and positive ion modes (ES^-) and (ES^+) with a 40 V cone voltage.

3. Results

3.1. γ-Degradation of flavonoids

Radiolysis of flavonoids in ethanol was performed with doses ranging from 0.2 to 20 kGy and concentrations ranging from 5×10^{-5} to 5×10^{-3} M. HPLC analysis showed a decrease of the peaks of galangin (retention time: $r_t = 34$ min), kaempferol ($r_t = 34$ min), quercetin ($r_t = 30$ min) and morin ($r_t = 28$ min), and concomitant apparition of new peaks indicating formation of several compounds. Fig. 1 shows chromatograms obtained for galangin, kaempferol, quercetin and morin (5×10^{-3} M) as controls, and for the 14 kGy-irradiated flavonol solutions. At this concentration and this irradiation dose, all flavonols were completely degraded except galangin (partially degraded), which has a non-substituted B-ring. For low concentrations ($< 10^{-4}$ M), 4 kGy was sufficient to cause complete disappearance of flavonol peaks. Similar results were recently obtained when quercetin was irradiated in methanol [23].

G1 ($r_t = 32$ min), K1 ($r_t = 32$ min), Q1 ($r_t = 28$ min) and M1 ($r_t = 33$ min) were detected as the major products formed during radiolysis of galangin, kaempferol, quercetin and morin, respectively. Using repeated reversed-phase semi-preparative HPLC, G1, K1, Q1 and M1 were separated from 5×10^{-3} M ethanol solutions prepared with 48 mg of galangin, kaempferol, quercetin and morin and irradiated with a dose of 14 kGy. Afterwards, eluent was evaporated at 45 °C under vacuum and gave 5.4 mg of G1, 11.6 mg of K1, 12.5 mg of Q1 and 8.4 mg of M1.

No degradation was observed after irradiation of flavones (luteolin, chrysin, apigenin), (±)-taxifolin, (±)-naringenin, (±)-eriodictyol and (−)-epicatechin. In this case, doses up to 20 kGy were tested with 5×10^{-3} M concentrations.

3.2. UV/Visible spectra of radiolysis products

Earlier studies concerning spectroscopic identification of flavonoids demonstrated that most flavones and flavonols exhibit two major absorption bands in the ultraviolet/visible region: Band I in the 320–385 nm range repre-

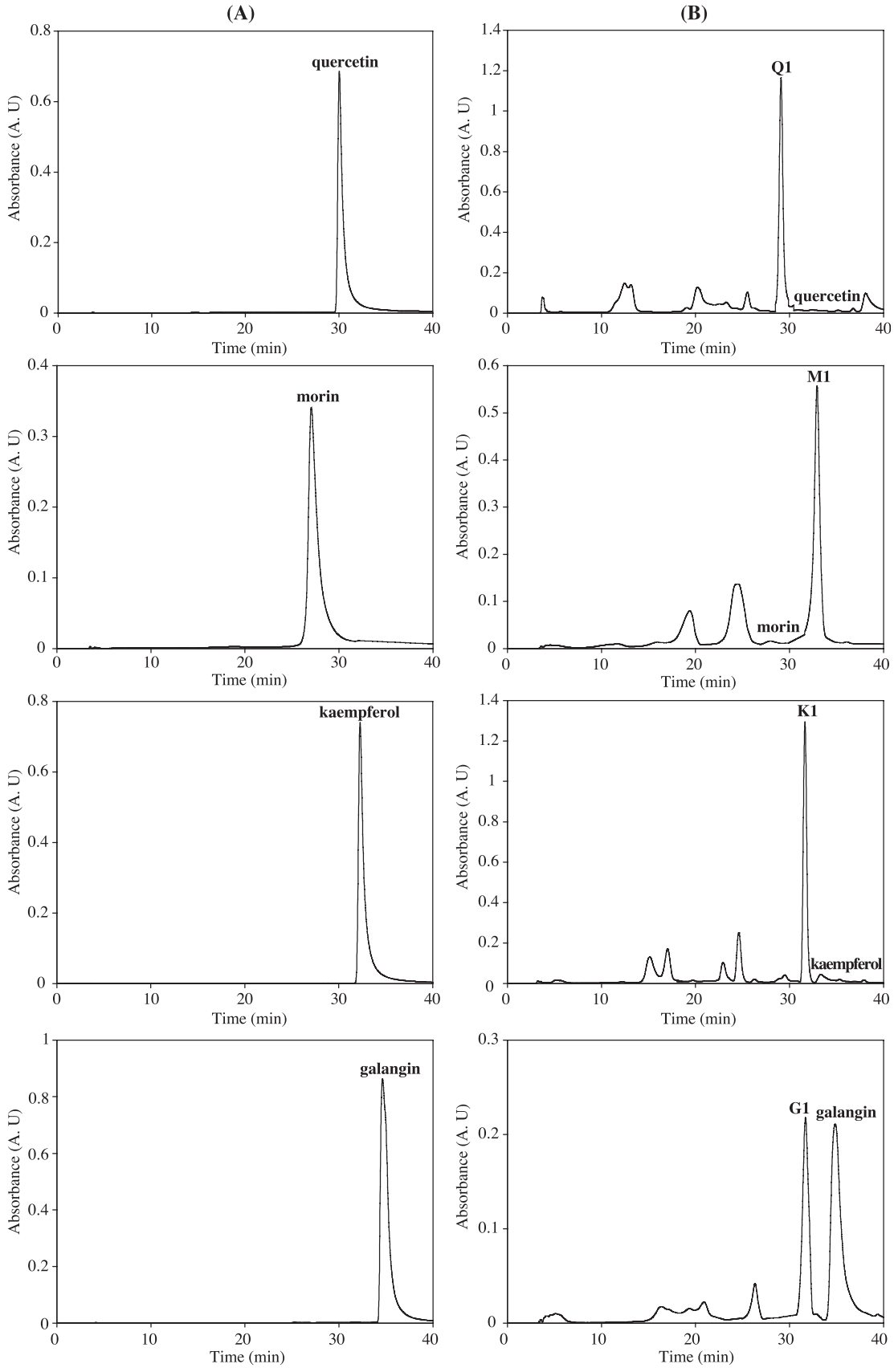

Fig. 1. HPLC chromatograms, recorded at 280 nm, of flavonol ethanol solutions (5×10^{-3} M), including quercetin, morin, kaempferol and galangin before irradiation (panel A), and after irradiation with a dose of 14 kGy (panel B).

senting the B–C ring absorptions, and Band II in the 240–280 nm range representing the A–C ring absorptions [24]. For flavonols, it has been observed that an increase in the number of B-ring hydroxyl groups induces a shift from 3 to 10 nm in Band I. For example, galangin (without any hydroxyl group), kaempferol (one hydroxyl in position 4′) and quercetin (3′, 4′-OH) have a maximal absorption at 359.5, 364.3 and 367.7 nm, respectively (Fig. 2).

After γ-degradation of galangin, kaempferol, quercetin and morin, we observed that the major radiolysis products (G1, K1, Q1 and M1) exhibit two absorption bands ranging from 270 to 298 nm and from 222 to 227 nm instead of Band I and Band II, respectively (Fig. 2). We concluded that the A–C and the B–C conjugations were destroyed, which indicated C-ring opening.

3.3. NMR and mass spectroscopic identification

Q1, M1, G1, and K1 were identified by ^{1}H, ^{13}C, COSY, HMQC and HMBC NMR and by negative and positive ES mass data.

3.3.1. Product Q1

The Q1 ^{1}H NMR spectrum consisted of (i) five aromatic protons (Table 1a): two meta coupled (*d*, $J=1.7$ Hz) at 6.18 ppm and at 6.10 ppm, and a series of three protons at 7.43 ppm (*d*, $J=1.6$ Hz), at 7.38 ppm (*dd*, $J=1.6$; 8.3 Hz) and at 6.79 ppm (*d*, $J=8.3$ Hz). These data indicated that the Q1 structure is composed of two rings, A and B, four and three substituted, respectively. (ii) Five aliphatic protons at 3.68 ppm (2H, *q*, $J=7.1$ Hz) and at 0.96 ppm (3H, *t*, $J=7.1$ Hz) representing an OCH_2CH_3 group.

The Q1 ^{13}C *J*mod spectrum consisted of: (i) five tertiary carbons at δ ppm (124.7, C-6′; 118.3, C-2′; 116.2, C-5′; 105.1, C-5; 101.9, C-3), (ii) 10 quaternary carbons identified as five O-linked aromatic carbons at δ ppm (168.4, C-2; 168.1, C-4; 155.4, C-6; 153.3, C-4′; 146.8, C-3′), two C-linked aromatic carbons at δ (120.7, C-1′; 105.4, C-1), one ketone group at 189.2 ppm (C-7) and two ester groups at 165.3 ppm (C-8/C-7′), (iii) two signals at 63.2 and 14.3 ppm corresponding to OCH_2 and CH_3 groups, respectively; this was consistent with the data obtained from the ^{1}H spectrum.

The tertiary carbon positions were deduced from the $^{1}H-^{13}C$ HMQC spectrum, and the contributions of

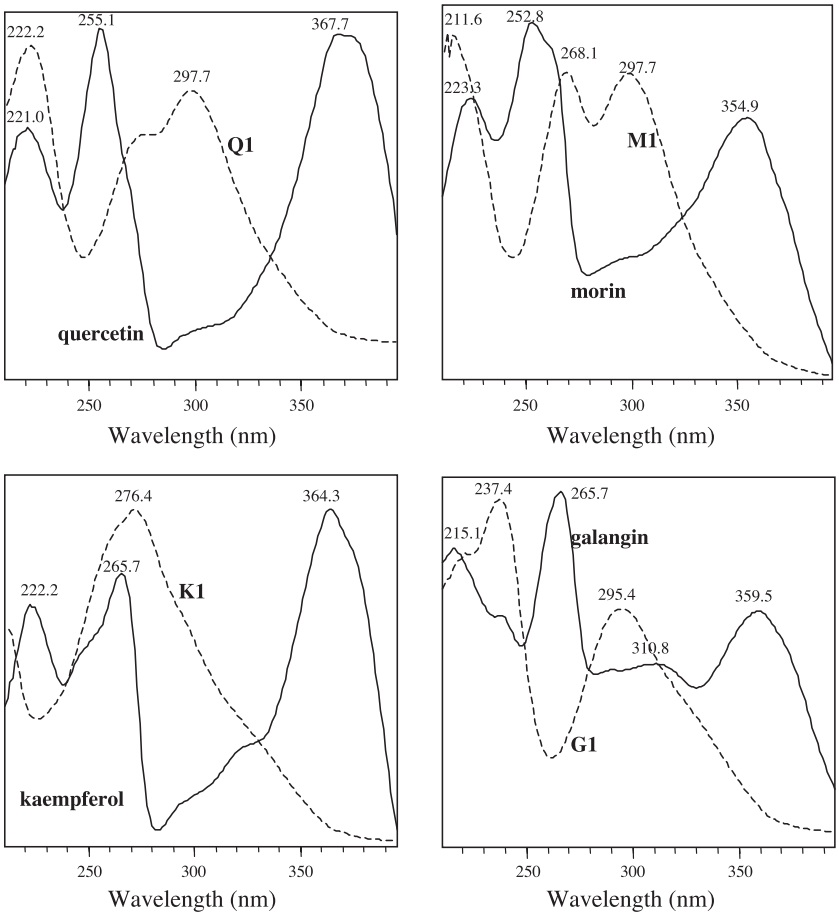

Fig. 2. UV spectra of flavonols: quercetin, morin, kaempferol and galangin, and their major radiolysis products: Q1, M1, K1 and G1, respectively.

chemical shifts to different O-linked and C-linked aromatic carbons were determined from carbon–proton long-range correlation observed on the HMBC spectrum (Table 1a).

The connection between rings A and B was established from the HMBC spectrum as follows: first, the fact that C-7′ was a quaternary carbon correlated with protons H-2′ and H-6′, and the fact that C-1′ was a C-linked carbon demonstrat-

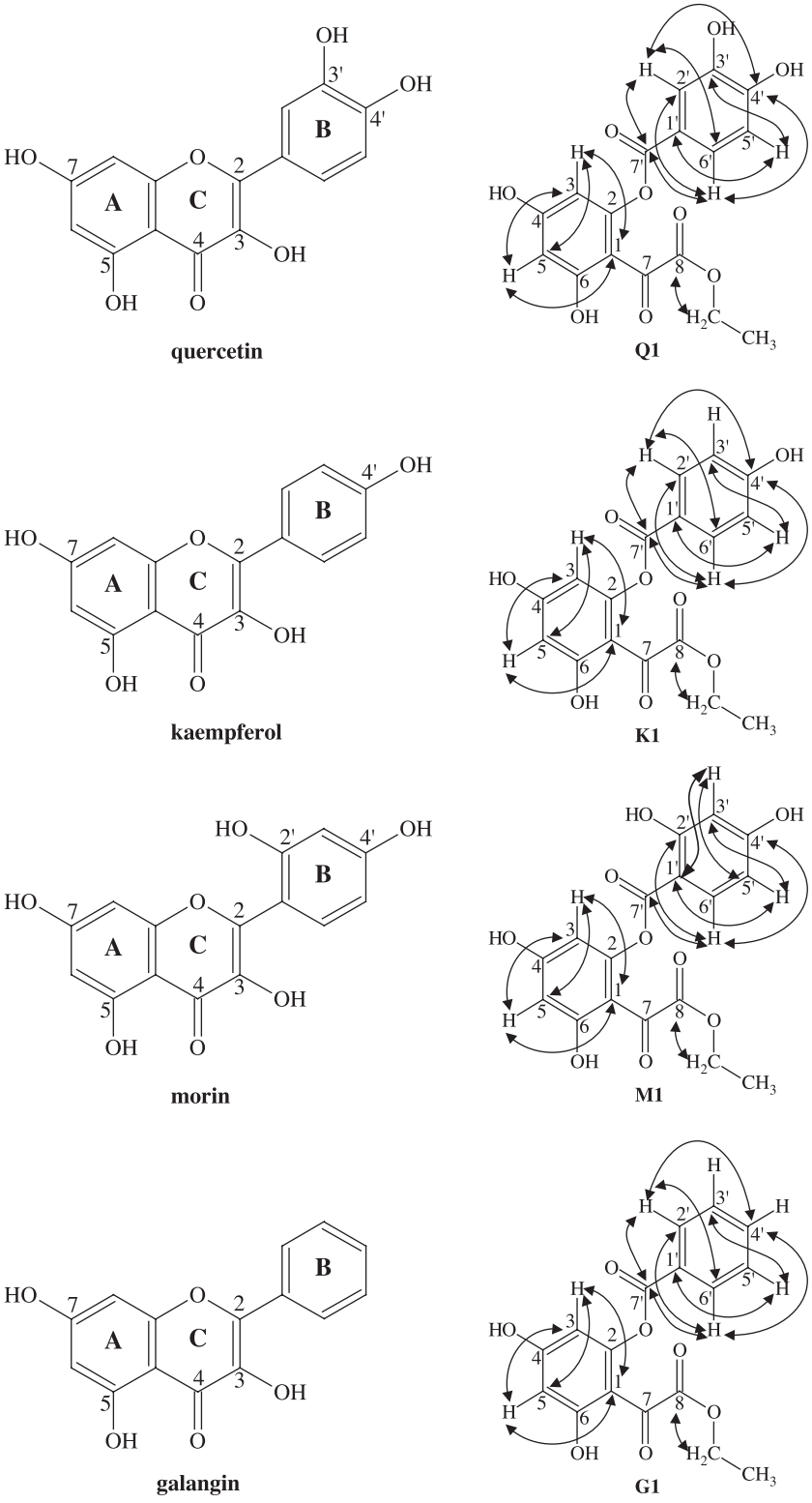

Fig. 3. Selected $^{1}H-^{13}C$ long-range correlations (^{3}J) (↔) observed in HMBC spectra of products Q1, K1, M1 and G1.

Table 1a
^{1}H (400 MHz); ^{13}C (100 MHz) NMR data and correlations observed in COSY, HMQC and HMBC spectra of products Q1 and K1 in CD_3OD/TMS (δ ppm; *J* Hz)

Position	^{1}H	COSY	^{13}C *J*mod/HMQC	HMBC
Product Q1				
1			105.4	
2			168.4	
3	6.18 *d* (1.7)	H-5	101.9	C-1; C-5
4			168.1	
5	6.10 *d* (1.7)	H-3	105.1	C-1; C-3
6			155.4	
7			189.2	
8			165.3[a]	
8-OCH_2	3.68 *q* (7.1)	CH_3	63.2	C-8
CH3	0.96 *t* (7.1)	8-OCH_2	14.3	
1′			120.7	
2′	7.43 *d* (1.6)	H-6′	118.3	C-4′; C-6′; C-7′
3′			146.8	
4′			153.3	
5′	6.79 *d* (8.3)	H-6′	116.2	C-1′; C-3′
6′	7.38 *dd* (8.3; 1.6)	H-2′; H-5′	124.7	C-2′; C-4′; C-7′
7′			165.3[a]	
Product K1				
1			105.1	
2			167.7	
3	6.19 *d* (2.2)	H-5	101.6	C-1; C-5
4			168.0	
5	6.10 *d* (2.2)	H-3	104.8	C-1; C-3
6			155.0	
7			188.7	
8			164.6[b]	
8-OCH_2	3.66 *q* (7.2)	CH_3	62.8	C-8
CH_3	0.95 *t* (7.2)	8-OCH_2	14.0	
1′			120.0	
2′	7.83 *d* (8.7)	H-3′	133.5	C-4′; C-6′ C-7′
3′	6.82 *d* (8.7)	H-2′	116.4	C-1′; C-5′
4′			164.8	
5′	6.82 *d* (8.7)	H-6′	116.4	C-1′; C-3′
6′	7.83 *d* (8.7)	H-5′	133.5	C-2′; C-4′; C-7′
7′			165.0[b]	

[a,b]Assignments may be interchangeable.

ed the C1′–C7′ linkage. Second, C-7′ was identified as an ester group, and C-2 as a non-hydroxylated O-linked carbon due to the asymmetrical structure of A-ring. This permitted demonstration of the C7′–O–C2 linkage. Therefore, connection between A and B rings was established through the C1′–C7′–O–C2 linkage.

The presence of a ketone group C-7 (189.2 ppm) and the quaternary C-linked carbon C-1 (105.4 ppm), together with the absence of long-range couplings on the HMBC spectrum between any proton and C-7 and the ester group C-8 (165.3 ppm), led us to deduce the C1–C7–C8 linkage. This was consistent with the OCH_2CH_3 position, which was illustrated by the ^{3}J correlation between the OCH_2 protons (3.68 ppm) and C-8. Finally, Q1 was identified as {2-[(3′, 4′-dihydroxybenzoyl) oxy]-4, 6-dihydroxyphenyl} (oxo) ethyl acetate (Fig. 3).

ES-MS analysis of Q1 gave molecular ions at *m/z* 361 $[M-H]^-$, at *m/z* 363 $[M+H]^+$ and at *m/z* 385 $[M+Na]^+$ in negative and positive spectra, respectively. This suggested 362 as a molecular weight for Q1 corresponding to the molecular formula $C_{17}H_{14}O_9$ and confirming the structure determined by NMR.

3.3.2. Products M1, G1 and K1

^{1}H spectra of these compounds (Tables 1a and 1b) were very similar compared to that of Q1. They differed from one another in the number of aromatic protons: five for M1, six for K1, and seven for G1. It was found that M1, K1 and G1 have one ring A (four substituted) and one ring B (three, two and mono-substituted, respectively).

^{13}C spectra of M1, K1 and G1 all gave 15 aromatic carbons and one OCH_2CH_3 group (Tables 1a and 1b). Positions of tertiary and quaternary carbons, and ethoxyl

Table 1b
^{1}H (400 MHz); ^{13}C (100 MHz) NMR data and correlations observed in COSY, HMQC and HMBC spectra of products M1 and G1 in CD_3OD/TMS (δ ppm; *J* Hz)

Position	^{1}H	COSY	^{13}C *J*mod/HMQC	HMBC
Product M1				
1			105.7	
2			167.6	
3	6.29 *d* (2.0)	H-5	102.0	C-1; C-5
4			168.1	
5	6.23 *d* (2.0)	H-3	105.1	C-1; C-3
6			155.4	
7			188.6	
8			165.5[a]	
8-OCH_2	3.90 *q* (7.2)	CH_3	63.5	C-8
CH_3	1.17 *t* (7.2)	8-OCH_2	14.2	
1′			104.5	
2′			167.2	
3′	6.37 *d* (1.6)	H-5′	103.8	C-1′; C-5′
4′			168.6	
5′	6.44 *dd* (8.0; 1.6)	H-3′; H-6′	109.8	C-1′; C-3′
6′	7.75 *d* (8.0)	H-5′	133.6	C-2′; C-4′; C-7′
7′			165.8[a]	
Product G1				
1			105.7	
2			167.5	
3	6.29 *d* (2.2)	H-5	101.9	C-1; C-5
4			168.0	
5	6.25 *d* (2.2)	H-3	105.0	C-1; C-3
6			155.1	
7			188.5	
8			165.6[b]	
8-OCH_2	3.78 *q* (7.1)	CH_3	63.3	C-8
CH_3	1.07 *t* (7.1)	8-OCH_2	13.9	
1′			130.1	
2′	8.10 *br d* (7.2)	H-3′	131.4	C-4′; C-6′; C-7′
3′	7.57 *br t* (7.8; 6.5)	H-2′; H-4′	130.0	C-1′; C-5′
4′	7.72 *br t* (6.4; 1.8)	H-3′; H-5′	135.5	C-2′; C-6′
5′	7.57 *br t* (7.8; 6.5)	H-4′; H-6′	130.0	C-1′; C-3′
6′	8.10 *br d* (7.2)	H-5′	131.4	C-2′; C-4′; C-7′
7′			165.6[b]	

[a,b]Assignments may be interchangeable.

groups were determined by procedures similar to those used for the Q1 identification.

ES-MS analysis demonstrated that the molecular formulas of M1, K1 and G1 were $C_{17}H_{14}O_9$ (molecular ions at *m/z* 361 $[M-H]^-$, at *m/z* 363 $[M+H]^+$ and at *m/z* 385 $[M+Na]^+$); $C_{17}H_{14}O_8$ (molecular ions at *m/z* 345 $[M-H]^-$, at *m/z* 347 $[M+H]^+$ and at *m/z* 369 $[M+Na]^+$); and $C_{17}H_{14}O_7$ (molecular ions at *m/z* 329 $[M-H]^-$, at *m/z* 331 $[M+H]^+$ and at *m/z* 353 $[M+Na]^+$), respectively.

Finally, M1, K1 and G1 were identified as {2-[(2′, 4′-dihydroxybenzoyl) oxy]-4, 6-dihydroxyphenyl} (oxo) ethyl acetate, {2-[(4′-hydroxybenzoyl) oxy]-4, 6-dihydroxyphenyl} (oxo) ethyl acetate, and {2-[(benzoyl) oxy]-4, 6-dihydroxyphenyl} (oxo) ethyl acetate, respectively (Fig. 3). We concluded that Q1, M1, K1 and G1 belonged to the same series of polyphenols named depsides.

4. Discussion

There is considerable interest in the role of HER in the toxic effects of ethanol, especially due to its implication in alcoholism-induced pathologies. However, it is not clear whether HER has a causal role in liver injury. It may contribute to mechanisms responsible for ethanol hepatotoxicity via: (i) HER/protein adduct formation (inducing immune response) [10,25], (ii) induction of mitochondrial permeability transition (which may play a role in ethanol-induced apoptosis) [10], (iii) oxidative stress and disturbance of the cellular pro-oxidant–antioxidant balance [11], (iv) interaction with cellular antioxidants (glutathione, ascorbic acid and α-tocopherol) [26] and with antioxidant enzymes (glutathione reductase, glutathione peroxidase, superoxide dismutase) [12]. Interaction of HER with cellular antioxidants has been described after observation of mitochondria antioxidant consumption [11,26].

HER was implicated in mitochondria permeability transition because ethanol and •OH radicals could not directly induce this effect. It has been speculated that HER, with a longer biological half life than •OH, may be especially effective in enhancing toxicity by diffusing to critical targets that are some distance from the production site of •OH. In a first step, •OH seems to react with ethanol to form HER, and secondly, HER contributes to the mitochondrial permeability transition [10].

In order to understand its mechanism of action and in order to propose strategy for treatment of alcoholic diseases using effective antioxidants and/or drugs able to reduce the generation of aggressive radical species, HER reactivity must be established, especially redox chemistry with antioxidants. In this study, we proposed to establish the reactivity scheme between HER and flavonoids using radiolysis for HER generation.

4.1. Implication of HER in radiolysis of flavonoid ethanol solutions

γ-Radiolysis of ethanol has been well studied [27,28]. Results obtained reported that in irradiated ethanol, the radiolytic species formed early are principally the solvated electron (e_s^-) and the H• atom via the following initial reaction:

$$CH_3CH_2OH \xrightarrow{\gamma} CH_3CHOH^+ + H^\bullet + e^-$$

The ejected electron loses its energy by successive ionizations and excitations. It becomes thermalized and then solvated by ethanol molecules:

$$e^- + CH_3CH_2OH \rightarrow e_s^-$$

These primary species react together and with solvent to form HER and other radiolytic products including H_2 and CH_3CHO according to the following reactions:

$$CH_3CHOH^+ + CH_3CH_2OH \rightarrow CH_3CHO + CH_3CHOH_2^+$$

$$e_s^- + CH_3CH_2OH_2^+ \rightarrow CH_3CH_2OH + H^\bullet$$

$$H^\bullet + CH_3CH_2OH \rightarrow H_2 + CH_3^\bullet CHOH$$

$$e_s^- + CH_3CHO \rightarrow CH_3CHO_s^-$$

$$CH_3CHO_s^- + CH_3CH_2OH_2^+ \rightarrow CH_3^\bullet CHOH + CH_3CH_2OH$$

Due to its reaction rate and its half-life, HER is well known for its reactivity with the solute in irradiated ethanol medium [29]. Based on information concerning its high reactivity [28] and according to our previous results [30], we suggested that HER was responsible for chemical degradation of quercetin, kaempferol, galangin and morin observed after their γ-irradiation in ethanol solutions. In the following sections, we will also discuss the role of solvated electrons.

4.2. Reactivity between flavonoids and HER

It is well established that the antioxidant activity of flavonoids is attributed in part to their reactivity with free radicals. Experimental studies demonstrated that the preferential primary redox sites for hydroxyl and superoxide attack are the 3-OH, 3′-OH and 4′-OH groups [19,31].

Three criteria for effective radical scavenging have been drawn for flavonoids: (i) the *ortho*-dihydroxyl structure in the B-ring, (ii) the 2,3-double bond which allows π electron delocalization from the B-ring, and (iii) the 3-OH and 5-OH with 4-oxo function in the A- and C-rings [32,33].

According to this antioxidant activity relationships, the redox site for the initial reaction with HER is probably 3-OH or B-ring. Radiolysis of luteolin, apigenin and chrysin (flavones analogues to quercetin, kaempferol and galangin,

respectively) showed that these flavones were not degraded. This result indicated that flavones were not able to scavenge HER, and therefore demonstrated the importance of the 3-OH group. Moreover, the major radiolysis products were formed after C-ring opening, whereas A- and B-ring structures were not affected by irradiation. This indicated that 5-OH, 7-OH, 2′-OH, 3′-OH and 4′-OH were not implicated in the transformation of quercetin, kaempferol, galangin and morin into Q1, K1, G1 and M1, respectively. However, the B-ring could be implicated in the formation of minor radiolysis products. For example, radicals formed during radiolysis of water reacted with flavonoids and led to formation of quinone in B-ring [33].

We also observed that taxifolin and (−)-epicatechin were not degraded after radiolysis in ethanol solution leading to the conclusion that the 2,3-double bond was important in the degradation of flavonoids into depsides. We concluded that the 3-OH group of flavonols was the primary redox site for HER scavenging and the 2,3-double bond was implicated in C-ring opening and finally formation of depside (Fig. 4).

We propose the following transformation mechanism of quercetin, kaempferol, galangin and morin into depsides Q1, K1, G1 and M1, respectively (Fig. 4). The first step is the H-transfer from 3-OH to HER leading to the flavonoxy ($Fl^{\bullet}$) formation. The second step is the reduction of the C2–C3 double bond followed by formation of a keto group in position 3 by a mesomeric effect (F11). Ethanol reacts with F11 and leads to formation of an intermediate product F12, which reacts again with HER to give the F13 radical. The following step is formation of a keto function in position 2 of F13 followed by formation of the F14 radical, which requires C-ring opening at C2–C3. The last step is the reaction of F14 with ethanol to form the depside D1 (Q1, M1, K1 or G1).

A similar mechanism has recently been proposed for reactions of quercetin and kaempferol with hydroxymethyl radicals ($^{\bullet}CH_2OH$), the major intermediate radical formed during methanol radiolysis [34,35]. Both flavonols reacted with $^{\bullet}CH_2OH$, and gave formation of two depsides identified as {2-[(3′, 4′-dihydroxybenzoyl) oxy]-4, 6-dihydroxyphenyl} (oxo) methyl acetate and {2-[(4′-hydroxybenzoyl) oxy]-4, 6-dihydroxyphenyl} (oxo) methyl acetate, respectively. Similarly, the 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) and the 2,6-di-*tert*-butyl-a-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxyl (galvinoxyl) radicals reacted with flavonols giving the corresponding depsides [36]. Consequently, HER, $^{\bullet}CH_2OH$, TEMPO and galvinoxyl play the same role because they all react with flavonols at the 3-OH group resulting in C-ring opening and depside formation.

Flavonoid reactivity with solvated electrons in aqueous media was also studied [37]. The data suggested that the C-4 keto group was the active site for redox attack by these species, whereas reactivity with the 3-OH group and the C2–C3 double bond seemed to be very unlikely. Therefore, in ethanol solution, flavonols may react with e_s^- to give minor radiolysis products. Because we were interested in major radiolysis products, these reactions were neglected.

4.3. Rate constants for flavonol reactions with her

HER reactivity was investigated using γ-radiolysis of flavonoid ethanol solutions. According to the above discussion and to the proposed mechanism of reaction (Fig. 4), reactions that must be taken into account for a kinetic scheme are:

$$\begin{aligned} &CH_3CH_2OH \xrightarrow{\gamma} e_s^-(G = 0.17\mu\ mol\ J^{-1}),\\ &CH_3CH_2OH_2^+(G = 0.17),\ H^{\bullet}(G = 0.24),\\ &CH_3CHO(G = 0.38), H_2(G = 0.19) \end{aligned} \quad (1)$$

$$e_s^- + CH_3CHO \rightarrow CH_3CHO^- + CH_3CH_2OH \quad (2)$$

$$CH_3CHO^- + CH_3CH_2OH_2^+ \rightarrow CH_3^{\bullet}CHOH + CH_3CH_2OH \quad (3)$$

$$H^{\bullet} + CH_3CH_2OH \xrightarrow{k_4} H_2 + CH_3^{\bullet}CHOH \quad (4)$$

$$CH_3^{\bullet}CHOH + CH_3^{\bullet}CHOH \xrightarrow{2k_5} (CH_3CHOH)_2 \quad (5)$$

$$Fl + CH_3^{\bullet}CHOH \xrightarrow{k_6} Fl^{\bullet}(F11) + CH_3CH_2OH \quad (6)$$

$$Fl^{\bullet} + CH_3CH_2OH \xrightarrow{k_7} F12 + H^{\bullet} \quad (7)$$

$$F12 + CH_3^{\bullet}CHOH \xrightarrow{k_8} F13(F14) + butan-2-ol \quad (8)$$

$$F14 + CH_3CH_2OH \xrightarrow{k_9} D1 + H^{\bullet} \quad (9)$$

Fl and D1 represent flavonols (quercetin, morin, kaempferol and galangin) and depsides (Q1, M1, K1 and G1), respectively. $Fl^{\bullet}$ denotes the flavonoxy radical formed after H-transfer from the 3-OH to HER.

In terms of radiation chemical yield, flavonol consumption and depside formation are governed by Eqs. (1) and (2), respectively.

$$aG(-Fl)\dot{D} = -\frac{d[Fl]}{dt} = k_6[Fl][HER] \quad (1)$$

$$aG(D1)\dot{D} = \frac{d[D1]}{dt} = k_9[F14][CH_3CH_2OH] \quad (2)$$

Where $\dot{D}$ (0.22 Gy/s) is the dose rate and a is the conversion coefficient ($a = \rho \times 10^{-6}$).

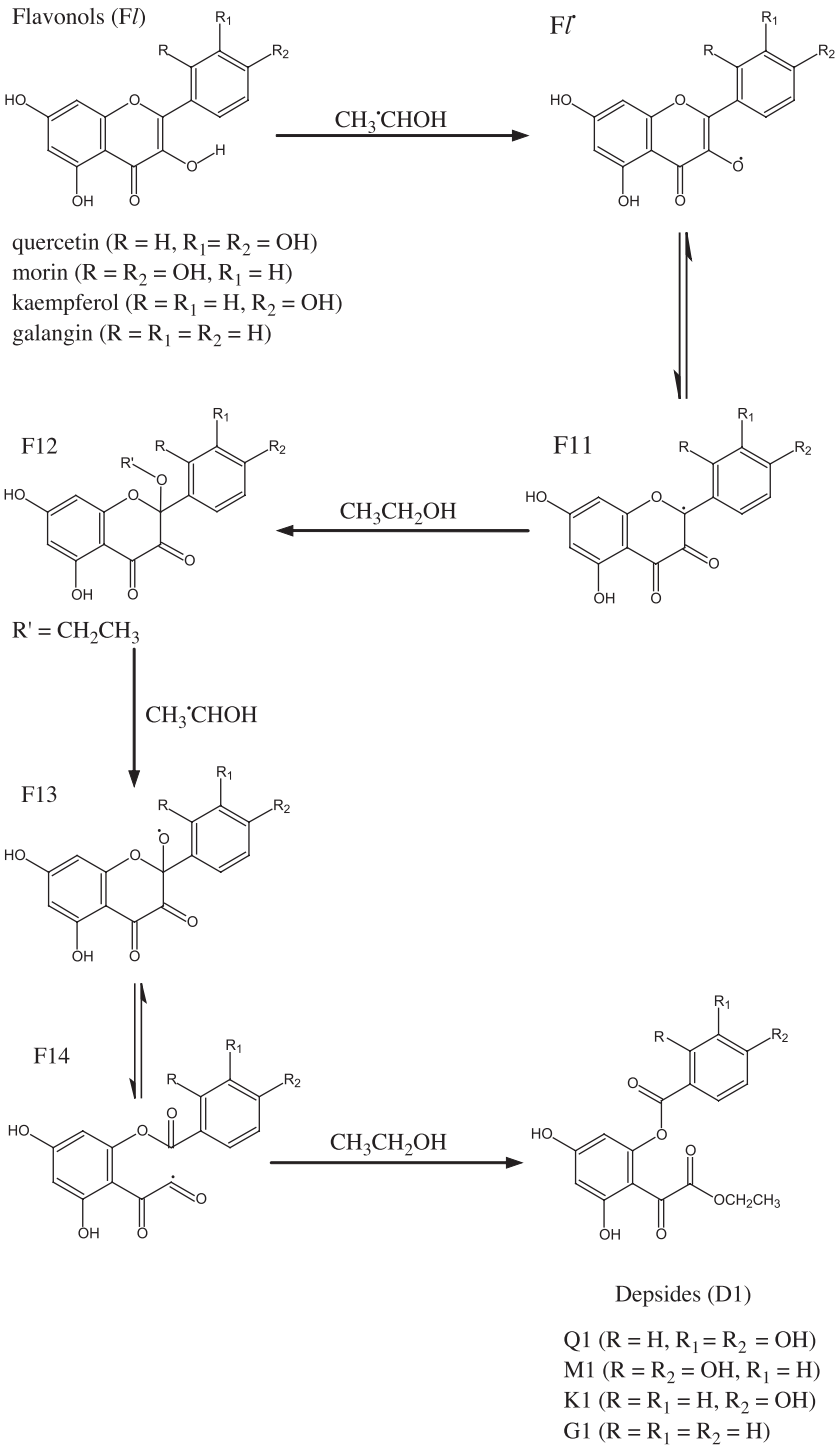

Fig. 4. Proposed transformation mechanism of flavonols into depsides.

HER steady-state concentration is the result of redox reactions, Eqs. (1)–(4) contribute to the increase in concentration, (Eqs. (5), (6) and (8) contribute to the decrease. The corresponding equation is:

$$k_6[\mathrm{Fl}][\mathrm{HER}] + k_8[\mathrm{F12}][\mathrm{HER}] + 2k_5[\mathrm{HER}]^2 = aG(e_s^-)\dot{D} + k_4[\mathrm{H}^\bullet][\mathrm{CH_3CH_2OH}] \quad (3)$$

$aG(e_s^-)\dot{D}$ is representative of reactions(1)–(3). k_5 was measured by Taub and Dorfman [27] and its value is 3.8×10^8 M^{-1} s^{-1}.

In the same manner, application of the steady-state principal to [H•] leads to:

$$k_4[\mathrm{H}^\bullet][\mathrm{CH_3CH_2OH}] = k_7[\mathrm{Fl}^\bullet][\mathrm{CH_3CH_2OH}] + k_9[\mathrm{F14}] \times [\mathrm{CH_3CH_2OH}] \quad (4)$$

Table 2
Radiation chemical yield of HER (G(HER)) and rate constants (k_6) for its reaction with flavonols

Flavonols	α	β	G(HER) (μmol J^{-1})	k_6 (M^{-1} s^{-1})
Quercetin	2.82	1.6×10^{-3}	0.73	4.0×10^{4}
Kaempferol	2.59	3.4×10^{-3}	0.69	3.1×10^{4}
Morin	2.61	2.5×10^{-3}	0.68	2.9×10^{4}
Galangin	2.35	4.8×10^{-3}	0.66	1.7×10^{4}

α and β are the calculated coefficients for linear regressions between G^{-1}(D1) and $[Fl]^{-1}$.

Expression of the radiation chemical yield of HER:

$$aG(\mathrm{HER})\dot{D} = aG(e_s^-)\dot{D} + k_4[H^\bullet][CH_3CH_2OH],$$

allows us to rewrite (Eq. (3)) as follows:

$$2k_5[\mathrm{HER}]^2 + k_6[\mathrm{Fl}][\mathrm{HER}] + k_8[\mathrm{F12}][\mathrm{HER}] = aG(\mathrm{HER})\dot{D} \quad (5)$$

To calculate G(HER) and k_6, we need k_8 and the [F12] values. For this, we consider that F13 radicals are completely transformed into F14 radicals by a mesomeric effect. The latter are unstable enough and do not accumulate during chemical reactions, which permits application of the quasi-steady state principle to [F14]:

$$\frac{d[\mathrm{F14}]}{dt} = 0$$

We obtain the following equation:

$$k_9[\mathrm{F14}][CH_3CH_2OH] = k_8[\mathrm{F12}][\mathrm{HER}] \quad (6)$$

Conjugation of Eqs. (2) and (6) gives:

$$aG(\mathrm{D1})\dot{D} = k_8[\mathrm{F12}][\mathrm{HER}] \quad (7)$$

Inserting this equation into Eq. (5), we obtain the HER concentration versus that of flavonols:

$$2k_5[\mathrm{HER}]^2 + k_6[\mathrm{Fl}][\mathrm{HER}] + a\dot{D}(G(\mathrm{D1}) - G(\mathrm{HER})) = 0 \quad (8)$$

The determinant of this equation is:

$$\Delta = k_6^2[\mathrm{Fl}]^2 - 8k_5a\dot{D}(G(\mathrm{D1}) - G(\mathrm{HER}))$$

Acceptable solution of Eq. (8) requires $\Delta>0$, i.e, $G(\mathrm{D1})<G(\mathrm{HER})$. Therefore, the HER concentration versus that of flavonols is given by the following equation:

$$[\mathrm{HER}] = \frac{-k_6[\mathrm{Fl}] + \sqrt{\Delta}}{4k_5} \quad (9)$$

Substituting this equation into Eq. (1), we obtain the dependence of the flavonol radiation chemical yields versus initial flavonol concentration:

$$G(-\mathrm{Fl}) = \frac{k_6^2[\mathrm{Fl}]^2}{4k_5a\dot{D}} \times \left(-1 + \sqrt{1 + \frac{8k_5a\dot{D}(G(\mathrm{HER}) - G(\mathrm{D1}))}{k_6^2[\mathrm{Fl}]^2}}\right) \quad (10)$$

Determination of k_6 and G(HER), characteristic parameters of the γ-degradation of flavonols in ethanol solution, demands resolution of Eq. (10). First, we determined concentrations of Q1, M1, K1 and G1 using calibration curves established with purified materials. Afterwards, we calculated the radiation chemical yields of quercetin, morin, kaempferol and galangin, and those of their major radiolysis products (Q1, M1, K1 and G1, respectively) from the following equation [38]:

$$G(\mu mol\ J^{-1}) = \frac{10^6}{\rho}\frac{\Delta M}{D} \quad (11)$$

Where D is the dose (Gy) and ΔM is the concentration (M) of formed or consumed molecules. Second, plotting radiation chemical yields of Q1, M1, K1 and G1 versus initial concentrations of quercetin, morin, kaempferol and galangin, respectively, we obtain the following empirical equations:

$$\frac{1}{G(\mathrm{D1})} = \alpha + \beta\frac{1}{[\mathrm{Fl}]} \quad (12)$$

α and β were calculated by linear regression, and they are summarized in Table 2.

In Fig. 5, we plotted the theoretical dependence curves of G(-quercetin), G(-morin), G(-kaempferol) and G(-galangin)

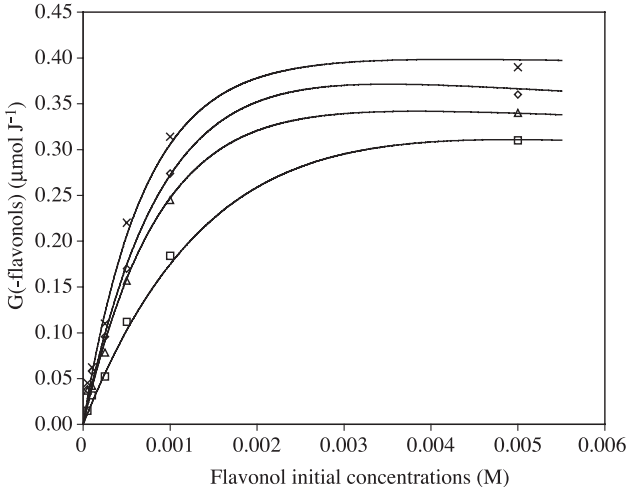

Fig. 5. Radiation chemical yields of flavonols versus their initial concentrations. Points represent experimental data (×: quercetin,◇: kaempferol, △: morin and □: galangin), and curves are the best fit of Eq. (10).

on initial flavonol concentrations. We observed that these theoretical curves fitted the experimental data very well, and the best fit gave the reaction constants between each flavonol and HER (Table 2).

We observed that quercetin ($k_6 = 4.0 \times 10^4$ M^{-1} s^{-1}) was the most efficient agent followed by kaempferol ($k_6 = 3.1 \times 10^4$ M^{-1} s^{-1}). Absence of 4′-OH greatly reduced the inhibition capacity because the rate constant of galangin ($k_6 = 1.7 \times 10^4$ M^{-1} s^{-1}) was three times lower than that of quercetin (Table 2). Morin, with two hydroxyl groups located in positions 2′ and 4′ in the B-ring, had an activity very similar to that of kaempferol. This indicated that hydroxylation of position 2′ were not important in HER scavenging. This is consistent with structure–activity relationships reported for flavonoids against superoxide and hydroxyl radicals [20–22,31,33].

In order to compare with standard molecules, superoxide dismutase (SOD) is considered as the first line of defense against the deleterious effects of oxygen radicals in cells by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and oxygen. Santiard et al. [39] demonstrated that this enzyme was capable of scavenging HER, and the rate constant for this reaction was calculated (k(SOD + HER) = 6.8×10^5 M^{-1} s^{-1}). Compared to the rate constants found for flavonols (Table 2), we concluded that flavonols are good HER scavengers.

Flavonoids are effective antioxidants largely distributed in diets rich in fruits and vegetables. This type of diet is well known to reduce numerous pathologies attributed in part to oxidative stress. It is important to determine whether flavonoids can also reduce liver injuries and consequently alcoholic diseases. During the last 10 years, numerous experimental studies concerning flavonoid metabolism helped to locate flavonoid absorption. The liver seems to be the principal organ involved [40]; large quantities of flavonoids are metabolized primarily in the liver, whereas small quantities may be metabolized by the intestinal mucosa [41]. Except for catechins, most of the flavonoids do not occur in plant as aglycones (molecules attached to sugar moities) but as flavonoid glycosides (flavonoids with sugar moities attached in the C3-position and, less frequently, the C7-position) [42]. The first step of in vivo metabolism is deglycosylation of these flavonoid glycosides, afterwards O-methylation, sulfonation and glucuronidation occur on the aglycone forms [40,43,44]. Evidence exists that flavonoids are transformed in the liver; for this reason, HER and aglycone forms of flavonoids could occur concomittantly in these tissues and the subsequent redox reactions could contribute to diminish oxidative stress due to chronic ethanol intoxication.

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