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Are the biological properties of kaempferol determined by its oxidation products?

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Abstract

Although flavonoid molecules have attracted considerable interest in recent years because of their antioxidant effect, there are considerable differences in their chemical properties. Electron paramagnetic resonance (EPR) spectroscopy was used to compare the oxidative free radical chemistry of two such molecules, kaempferol and luteolin, which have the same empirical formula but differ in the position of one OH group. Whereas the basic flavonoid structure remains intact in luteolin, structural changes occur in kaempferol after one-electron oxidation. Autoxidation of kaempferol in alkaline solution and oxidation by $O_2^{\cdot-}$ at pH 7 led to rapid fragmentation. In contrast, oxidation by horseradish peroxidase/hydrogen peroxide, xanthine/xanthine oxidase (X/XO) or a Fenton reaction system produced a radical whose structure appeared to be based on dimerisation of either the original or a fragment of the flavonoid. Hence, the biological properties of kaempferol are likely to be determined by the chemistry of its oxidation products.

Keywords: *Kaempferol, luteolin, EPR, oxidation, free radical*

Introduction

Flavonoid molecules have attracted considerable interest in recent years, because of their antioxidant properties, and their occurrence in plants that are used as traditional medicines in many parts of the world. Based on measurements of antioxidant activity and free radical scavenging ability, individual flavonoid molecules are now being used as dietary supplements with claims for anti-aging and disease prevention properties. However, it is by no means clear that such molecules consumed individually will have the same biological effects as when part of a whole food. Nor is it clear whether the biological properties are determined by the individual flavonoid molecules, or by their reaction products.

In plants there are large numbers of flavonoid molecules, and there are likely to be considerable differences in their chemistry depending on the nature and position of substituent groups in the basic flavonoid structure. From a dietary supplement/herbal medicine point of view, it is especially important to understand their behaviour with respect to oxidation, since their use is being promoted on the basis of their antioxidant

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properties. The present paper compares the oxidative free radical chemistry of luteolin and kaempferol, two molecules, which differ only in the position of one OH group. On the basis of these results, we propose that the biological activity of kaempferol is likely derived from oxidation products.

The structures of luteolin and kaempferol are shown in Figure 1a,b. Each has two aromatic rings, one heterocyclic ring and four substituent hydroxyl groups; ring C contains also a keto group. Luteolin is a flavone with a catechol group in ring B. Kaempferol, a flavonol, has the OH group at carbon 3′ of the B ring replaced by one at carbon 3 of the C ring. Various measurements of the antioxidant activity did not produce a clear picture as to which of the two compounds has the higher free radical scavenging activity, e.g. peroxyl radicals and azide radicals were better scavenged by kaempferol, but galvinoxyl radicals were scavenged from both flavonoids with equal intensity [1–3].

Autoxidation proceeds by deprotonation and electron abstraction to produce ionised radical anions. This was performed using alkaline, aerated solutions to produce initial information on the oxidised components. In addition, reactions with three aqueous oxidation systems were investigated at pH 7. These were: (a) horseradish peroxidase and hydrogen peroxide (HRP/H_2O_2), which has been reported to form three complexes with a high oxidation potential [4]; (b) xanthine and xanthine oxidase (X/XO), which is supposed to generate superoxide anion radicals [5,6]; and (c) the Fenton reaction system, which generates hydroxyl radicals [7]. Reactions with $O_2^{\cdot-}$ were also investigated directly using potassium superoxide at pH 6.9.

Materials and methods

The flavonoids kaempferol (≥96% purity) and luteolin (≥99% purity) and the enzyme horseradish peroxidase (~150 units/mg) were purchased from Fluka (Vienna, Austria), catalase (from bovine liver, 2000–5000 units/mg), XO (from buttermilk, 22 units/ml) and superoxide dismutase (SOD) (from bovine erythrocytes, 2500–7000 units/mg) were purchased from Sigma (Vienna, Austria), potassium

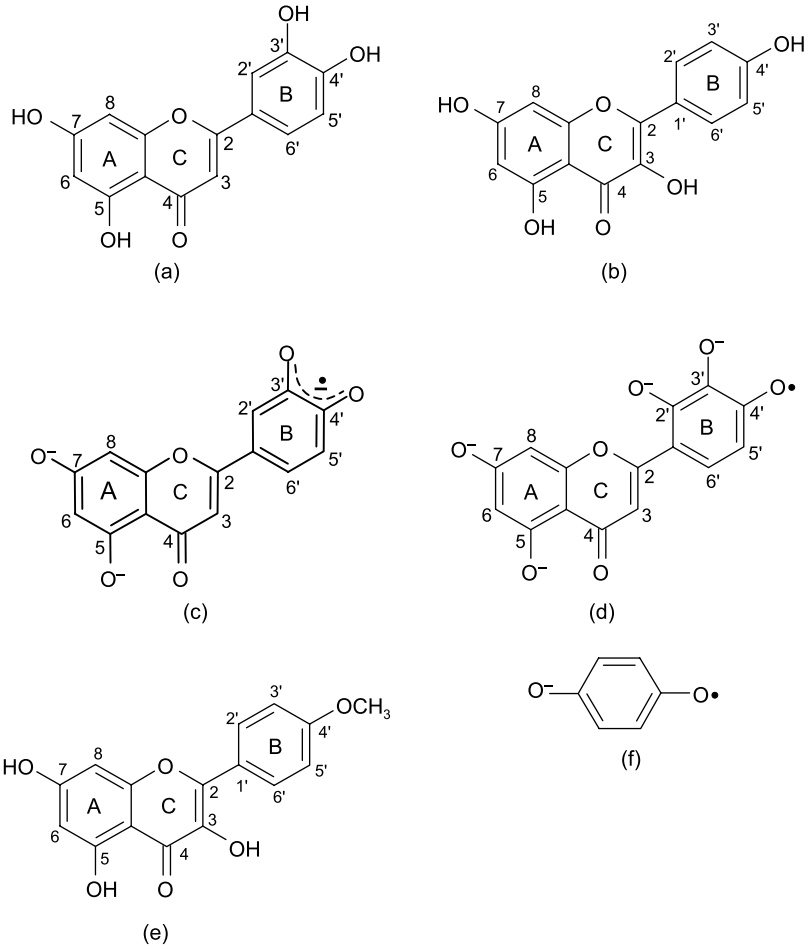

Figure 1. Chemical structures of (a) luteolin; (b) kaempferol; (c) the initial radical from the oxidation of (a); (d) possible second radical from the oxidation of (a); (e) kaempferide; (f) *p*-benzosemiquinone.

superoxide from Aldrich (Vienna, Austria), and kaempferide from Extrasynthese S.A. (Lyon, France).

Autoxidation procedures

Two different experimental setups were used in order to investigate the influence of oxygen levels as well as of the relative concentrations of NaOH and the phenol. In both procedures a 1 mM stock solution of the phenol in DMSO was used.

In the first procedure (Proc. 1), 200 µl of a 0.1 M sodium hydroxide solution were transferred into an electron paramagnetic resonance (EPR) flat cell (Wilmad-Labglass, Buena, NJ, USA), then 200 µl of the flavonoid solution were added. The reaction between the flavonoid and the alkali was immediately visible as a strong yellow band at the interface of the two liquid phases. This then spread slowly through the cell as a result of diffusion of the molecules. After addition of the flavonoid, the flat cell was immediately placed in the spectrometer and the spectrum recorded within 1 min. In this procedure the amount of oxygen is limited to that dissolved in the solutions. The second procedure (Proc. 2) was carried out in an Eppendorf tube to test the influence of a higher oxygen concentration. Two hundred microliter of the NaOH-solution and 200 µl of the flavonoid solution were transferred into an Eppendorf tube and mixed with a vortex mixer for a few seconds. The mixed solution had an intensive yellow colour and since the oxidation is an exothermic process the tube walls got warm very quickly. The oxidised solution was transferred into a flat cell as quickly as possible and placed immediately into the spectrometer for recording the spectrum. The oxygen concentration in this experiment (Proc. 2) should have been much higher than in the first set up (Proc. 1).

The dependence of relative concentrations of NaOH and the flavonoid was tested using Proc. 2 by thorough mixing of the two solutions in an Eppendorf tube. The two stock solutions were combined in different amounts; 1:1, 1.67:1 and 3:1 volume ratios of flavonoid:NaOH solutions.

Oxidation with horseradish peroxidase/hydrogen peroxide (HRP/H_2O_2)

Concentrations of solutions for the system HRP/H_2O_2 were similar to those of Miura et al. [8]. Two hundred microliter of a potassium phosphate buffer (pH 7) were mixed in an Eppendorf tube together with 50 µl of $ZnCl_2$ (10 mM), 50 µl HRP (12.5 µM), and 5 µl H_2O_2 (100 µM). All solutions were made with distilled water. Two hundred microliter of the mixed solution were transferred into an EPR flat cell and 200 µl of the flavonoid stock solution were added.

Oxidation with xanthine/xanthine oxidase(X/XO)

The system X/XO was used to generate superoxide anion radicals. The experiment was carried out with 200 µl of a potassium phosphate buffer (pH 7) mixed for a few seconds together with 20 µl of xanthine solution (657 µM) and 5 µl of XO solution (8 µl XO/1 ml). All individual solutions were prepared with distilled water. Two hundred microliter of the mixed solution were transferred into the flat cell and 200 µl of the flavonoid stock solution were added.

Oxidation in a Fenton reaction system

Hydroxyl radicals are generated in the Fenton system by the reaction of H_2O_2 with Fe(II). The reagent concentrations were those of Blank et al. [9], except that ascorbic acid was omitted in the present work. Two hundred microliter of a potassium phosphate buffer (pH 7) were placed in an Eppendorf tube. Five microliter of $FeCl_3 \cdot 6H_2O$ (10 mM), 5 µl EDTA (25 mM) and 5 µl H_2O_2 (100 µM) were added and the solution was mixed for a few seconds using a vortex mixer. Distilled water was used for preparation of the individual solutions. Two hundred microliter of the mixed solution were transferred in the flat cell and 200 µl of the flavonoid stock solution were added.

In each case the flat cell was placed in the spectrometer within 1 min and the EPR spectrum recorded immediately after tuning the spectrometer.

Potassium superoxide

The reaction of superoxide radical anions with phenols was investigated using potassium superoxide. For this purpose 40 µl of the flavonoid solution (10 mM) were mixed in an Eppendorf tube with 60 µl of distilled H_2O in the case of luteolin and with 160 µl DMSO in the case of kaempferol. The solution was added to c. 0.5 mg KO_2 and the reaction was stopped after approximately 5 s by adding a pH 6.9 buffer solution (300 µl for luteolin, 200 µl for kaempferol) containing 5 µl catalase (1 mg/ml) and 10 µl SOD (2 mg/ml). Distilled water was used for the preparation of the individual solutions. The resulting solution was then transferred into a flat cell which was placed in the spectrometer and the spectrum was recorded as quickly as possible.

EPR spectroscopy

All EPR spectra were acquired in 1024 points using a Bruker ESP 300E CW spectrometer operating at X-band frequencies and equipped with an ER4103TM cavity. Microwave generation was by a klystron and the microwave frequency recorded continuously with a frequency counter. Hundred kilohertz of modulation frequency and 20 mW microwave power were used for

all measurements. Magnetic field sweep widths were in the range 0.9–2.0 mT, depending on the spectral widths. For most measurements a modulation amplitude of 0.01 mT was used and the spectra accumulated in ten scans. Where different conditions were used, these are indicated in the relevant figure captions.

After placing the flat cell in the microwave cavity, the spectrometer was tuned manually to minimise the time between commencement of the reaction and recording the spectrum.

All of the parameters derived from the spectra in this work were confirmed by simulation using the Bruker Simfonia software. However, simulations are only shown for the more complex spectra. *g*-values are expressed relative to the diphenylpicrylhydroxyl (DPPH) ($g = 2.0036$) which was used as an external standard.

Results and interpretation

Luteolin

Autoxidation of luteolin (Figure 1a) gave two EPR signals at different times after starting the reaction. The spectrum of the first radical (Figure 2a), which is similar to that reported by Kuhnle et al. [10], Cotelle et al. [11] and van Acker et al. [12] can be simulated by four inequivalent proton couplings (Figure 2b). This subsequently developed into an eight peak spectrum from three inequivalent proton splittings (Figure 2c), although the first radical was stabilised if $ZnCl_2$ was added to the alkali solution. The *g*- and hyperfine parameters for these spectra (all of which were confirmed by simulation) are presented in Table I.

Table I. Summary of the hyperfine splittings and *g*-values observed after oxidation of luteolin and kaempferol.

Figure	a(^{1}H) (mT)	*g*-value
2a,b	0.275 0.150 0.125 0.117	2.0047
2c	0.465 0.083 0.025	2.0048
3a	0.238 × 4	2.0047
3b	0.088 × 8	2.0049
3c,d	0.095 × 4 0.080 × 4	2.0049
4b,c	0.108 × 2 0.075 × 4	2.0049
4d	0.324 0.143 0.087	2.0048
5a–d	0.110 × 2 0.105 0.100 × 2	2.0046

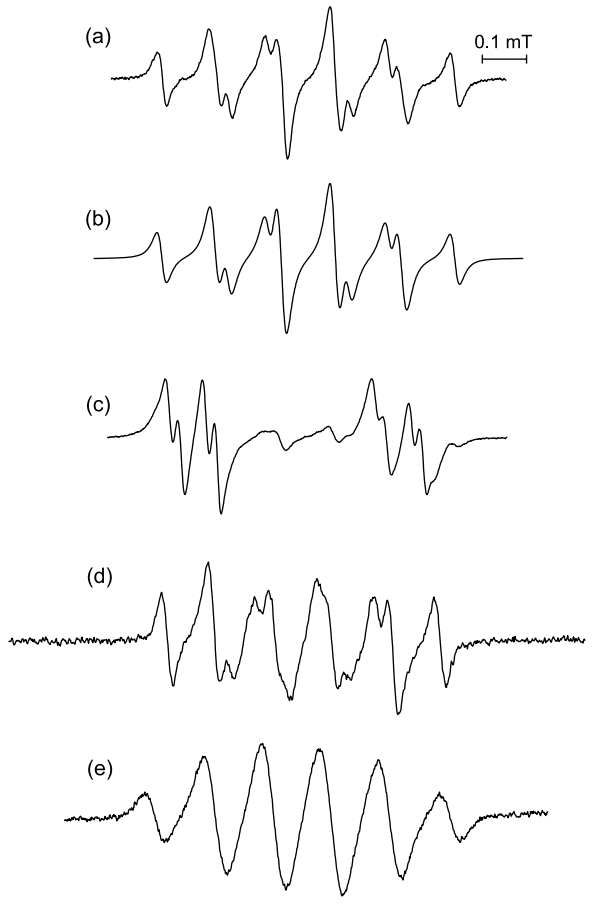

Figure 2. EPR spectra of (a) the first radical from autoxidised luteolin (3 scans); (b) its simulation; (c) the second radical from autoxidised luteolin; (d) after oxidation with KO_2; (e) after oxidation with X/XO.

Oxidation of luteolin with HRP/H_2O_2, X/XO, the Fenton reaction system or potassium superoxide at pH 7 all resulted in a 6 peak spectrum similar to Figure 2a. However, with potassium superoxide the spectrum consisted of sharper lines (Figure 2d) than those obtained with HRP/H_2O_2, X/XO, and the Fenton reaction system (Figure 2e). Resolution of these latter spectra was not improved by decreasing the modulation amplitude or by flushing the solutions with N_2 gas, so the different line widths would appear to be genuine and not artefacts of the experimental conditions. However, beyond the fact that the systems which gave the broader line spectra contained transition metals, we have no explanation for the line width differences.

The oxidation site in luteolin is most likely the catechol group on ring B, which is considered to be the region responsible for the antioxidant activity in flavonoids [13]. The probable structure of the radical from oxidised luteolin is shown in Figure 1c. The largest proton splitting can be assigned to the proton on carbon 6′, whereas smaller splittings around 0.1 mT come from interactions with protons in positions 2′, 5′ and 3 [11].

The second radical, which was detected at pH 13 after the first radical decreased in intensity, was also reported

by Cotelle et al. [11]. These authors explained the structure as a result from oxidation at carbon 2′ (Figure 1d). With this radical, the largest hyperfine splitting again comes from the proton on carbon 6′ and the smaller splittings from protons on carbons 5′ and 3.

Kaempferol

Autoxidation of kaempferol led to five distinct EPR signals, along with additional weak peaks from components that could not be fully resolved. Thus the free radical chemistry of kaempferol is considerably more complex than that of luteolin. The initial signal obtained with equal volumes of the kaempferol and NaOH solutions was a five peak spectrum (Figure 3a), consistent with four equivalent ^{1}H atoms in the radical. When there was adequate O_2 supply, this changed rapidly to a septet spectrum (Figure 3b), which was best simulated with eight equivalent ^{1}H atoms (the two outermost peaks being too weak to observe in the EPR spectrum). Changing the kaempferol:NaOH ratios resulted in different signals. With a 1.67:1 ratio, additional structure was observed on the septet signal after the quintet had decreased (Figure 3c); this spectrum was simulated with two sets of four equivalent ^{1}H atoms (Figure 3d). A 3:1 ratio kaempferol:NaOH gave a spectrum with at least three components (Figure 4). The first spectrum (Figure 4a) contained the quintet and a signal with 13 peaks (compound 2). Approximately 5 min later the quintet had disappeared and a mixture of two signals was visible, including the 13 peak spectrum (Figure 4b). After another c. 21 min compound 2 had almost disappeared and a spectrum of eight peaks (compound 3) with nearly equal intensity (Figure 4d) was observed. In order to produce the simulation of compound 2 (Figure 4c), a small amount of the simulation of compound 3 was first subtracted from Figure 4b, since this component was already present in the spectrum.

When kaempferide, which has a similar composition to kaempferol except that the hydroxyl group on ring B is replaced by a methoxy-group (Figure 1e), was used for the autoxidation experiment, no spectrum was observed, thus supporting the assumption that the oxidation site in kaempferol is the hydroxyl group on ring B.

Oxidation of kaempferol at pH 7 with HRP/H_2O_2, X/XO, or the Fenton reaction system gave the same EPR spectrum in each case (Figure 5); this could be simulated with five proton couplings around 0.1 mT. The spectrum (Figure 5a) shows the presence of a small amount of the quintet signal, which was seen with all the autoxidation procedures. Oxidation with

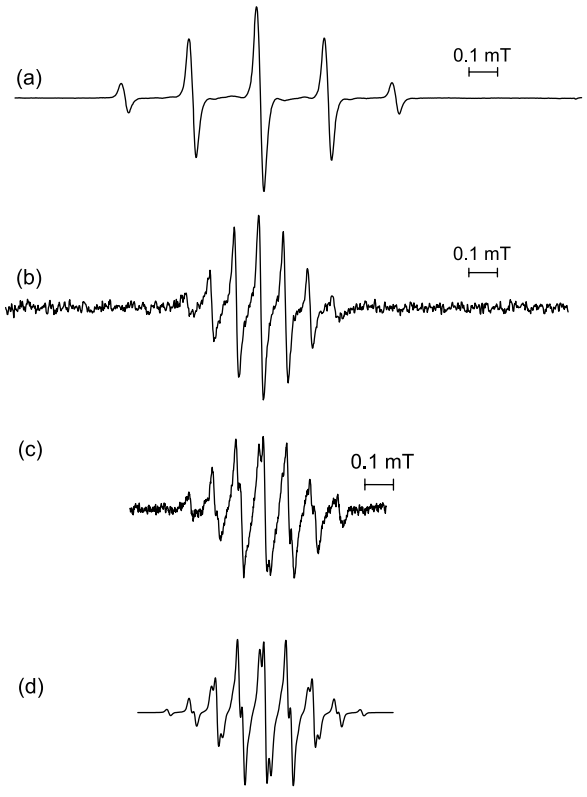

Figure 3. EPR spectra of autoxidised kaempferol (a) the initial radical (observed under conditions of limited O_2) and 1:1 ratio kaempferol:NaOH solutions (MA 0.05 mT, 1 scan); (b) the 2nd radical (obtained under O_2-rich conditions, or after a time delay when O_2 was limited and 1:1 ratios kaempferol:NaOH solutions); (c) using a kaempferol:NaOH = 1.67:1 ratio; (d) a simulation of (c).

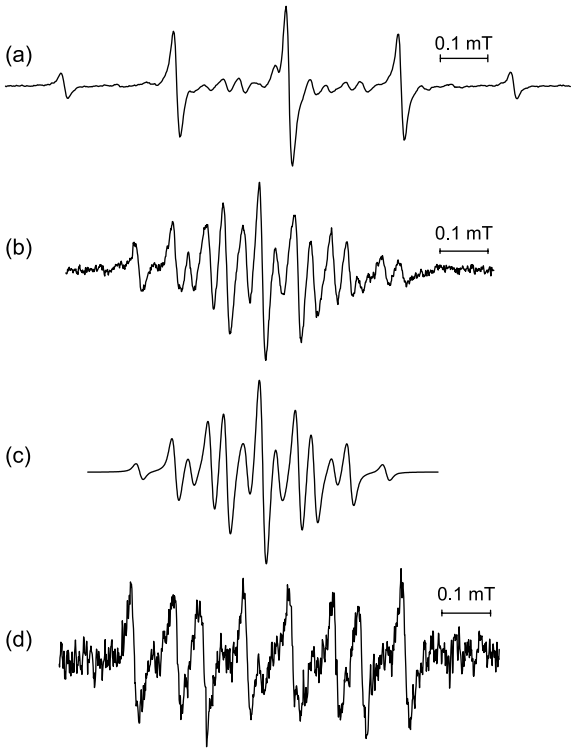

Figure 4. EPR spectra obtained from autoxidation of kaempferol using a 3:1 kaempferol:NaOH ratio (a) the initial spectrum (3 scans); (b) the spectrum obtained 5 min after (a); (c) a simulation of (b) after subtracting a small contribution from (d) which was obtained 21 min after (a).

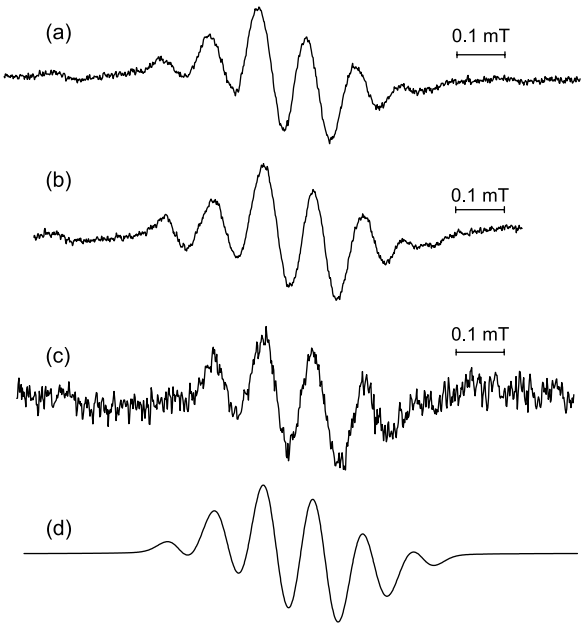

Figure 5. EPR spectra from kaempferol after reaction with: (a) xanthine/xanthine oxidase (MA 0.08 mT); (b) HRP/H_2O_2 (MA 0.05 mT, 20 scans); (c) the Fenton reaction system (MA 0.1 mT, 20 scans); (d) their simulation.

potassium superoxide at pH 6.9 resulted only in a quintet spectrum that is similar to Figure 3a. The *g*- and hyperfine parameters for these spectra (all of which were confirmed by simulation) are presented in Table I along with the results obtained with luteolin.

The EPR spectral parameters for the initial free radical signal seen on autoxidation of kaempferol are identical to those of *p*-benzosemiquinone (Figure 1f) [10]. It appears, therefore, that there is rapid fragmentation of the flavonoid structure. According to the spin distribution calculations of van Acker et al. [12], the unpaired electron density in the kaempferol radical is mainly in ring B and is largely associated with the oxygen atom from which the proton was removed. These authors also measured the half peak oxidation potential of kaempferol in the pH range 2–13 and showed that no further release of protons occurred at pH > 9.

The "7 peak spectrum" (Figure 3b) was best simulated by eight equivalent protons with 0.088 mT hyperfine splitting. The oxidised solution was deep yellow, indicating the presence of *p*-benzoquinone. A potential role for the *p*-benzosemiquinone radical in the formation of the radical responsible for this spectrum was demonstrated by oxidising an alkaline solution of phenol with a trace of hydroquinone in a method analogous to Proc. 2. Its spectrum was identical to that in Figure 3b. The same signal was also observed after oxidation of a pure phenol solution but the intensity was much lower than from the solution containing a trace of hydroquinone, thus suggesting that the radical reaction is catalysed by hydroquinone or one of its oxidation products (*p*-benzosemiquinone).

Hydroquinone and quinone may form a charge-transfer complex called quinhydrone. Although this complex seems to be more stable in a weakly alkaline solution of pH 8.0 [14], it is likely not the substrate for the radical forming the seven peak spectrum. Quinhydrone has a purple colour, whereas the oxidised kaempferol solution at pH 13 had an intensive yellow colour consistent with the formation of benzoquinone.

The EPR spectrum obtained with a kaempferol to NaOH ratio of 1.67:1 (Figure 3c) could be fitted by two sets of four equivalent protons (Figure 3d) with an average hyperfine splitting the same as that in Figure 3b. It is probable, therefore, that this signal is also derived from a similar structure to that responsible for the seven peak spectrum, and that both of these contain two aromatic rings. At the present time, however, it has not been possible to assign specific structures to these radicals.

The two additional signals that were seen with a kaempferol:NaOH ratio to 3:1 are probably also radicals generated from breakdown products of kaempferol. The first of these (Figure 4b) was simulated with six proton couplings (Figure 4c), four equivalent with a smaller value than the other two. The second signal (Figure 4d), which was detected after the previous one decreased in intensity, had three inequivalent protons. No structures are suggested for these radicals at the present time.

Oxidation of kaempferol with HRP/H_2O_2, X/XO, or the Fenton reaction system at physiological pH of 7 resulted in a spectrum with a sextet structure from interaction of the unpaired electron with five ^1H nuclei. In addition, a weak quintet signal from *p*-benzosemiquinone indicated that some degradation of kaempferol also occurred. No radical was detected when oxidation of kaempferide (Figure 1e) by HRP/H_2O_2 was attempted, thus indicating that the OH-group on ring B is involved in the oxidation of kaempferol. The generation of a phenoxyl derivative where the OH-group in ring B is oxidised can be excluded because of the small hyperfine splitting constants around 0.1 mT. For para-substituted phenoxyl radicals, hyperfine splittings for the two hydrogens on carbon 3′ and 5′ would be expected to be in the range 0.4–0.7 mT dependent on the substituents [15]. The small splittings in the present experiments suggest a dimeric or polymeric structure, in which the unpaired electron spin density is delocalised over two aromatic rings and is, therefore, smaller at individual carbon atoms.

Discussion

Oxidation of kaempferol under different conditions led to the detection of six different radicals. One of them, the initial quintet in alkaline solutions, was published by Kuhnle et al. [10] and assigned to *p*-benzosemiquinone,

whereas the other signals have not been published previously. Van Acker et al. [12] also detected a quintet after autoxidation in KOH, but reported different hyperfine splitting constants to those we obtained here. Additionally, these authors reported the formation of a weak triplet when HRP/H_2O_2 at pH 7 was used as the oxidising agent; we believe that this could be part of the sextet spectrum which we detected with HRP/H_2O_2.

The autoxidation experiments showed a strong dependency of the radical chemistry on the amount of oxygen and on the ratio of kaempferol to NaOH. This could be because the radical chemistry is most likely based on dimeric or polymeric structures of degradation products of kaempferol. The *p*-benzosemiquinone radical was also detected in the oxidation experiments at pH 7 (with KO_2, HRP/H_2O_2, X/XO and the Fenton reaction system), which again shows that degradation of the kaempferol structure occurred, and suggests that further radical reactions may be based on such oxidation products. Yu et al. [16] reported the formation of dimers from phenoxyl radicals in water after oxidation of phenol with HRP/H_2O_2, and the majority of the spectra produced by kaempferol oxidation are consistent with the generation of di- or polymeric structures from the phenol that is generated by fragmentation of the flavonoid molecule. This assumption is also supported by the small values for the ^{1}H hyperfine splitting constants in the spectra.

If the formation of a phenoxyl derivative on ring B is the initial product of oxidation, as suggested by van Acker et al. [12], the rapid appearance of the EPR signal from *p*-benzosemiquinone indicates that this radical is unstable and fragmentation occurs at the 2–1′ bond. Water addition to the double bond is a likely first step after oxidation of ring B (Figure 6a). This 2,3-dihydroxyflavanon has been reported by Frey-Schröder and Barz [17]. 2,2-Dihydroxy-1-(2,4,6-trihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propandion, which is formed when ring A is opened, has been detected by Miller and Schreier [18] (Figure 6b). These authors also detected the formation of 4-hydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid from the oxidation of kaempferol.

In the reaction of HRP with H_2O_2, three complexes with high oxidation potentials are formed [4]. Two of these (complexes II and III) can be reduced directly to the ferric form of the enzyme, whereas complex I is first transformed into complex II. There are previous reports of products of the reaction of the HRP/H_2O_2 system with kaempferol, but it is difficult to relate the results of these investigations to the present results. For example, Miller and Schreier [18] identified several products using UV-, IR-spectroscopy, mass spectrometry, ^{1}H-NMR and ^{13}C-NMR, but none of these had structures that could be related to the hyperfine structure in our EPR spectra.

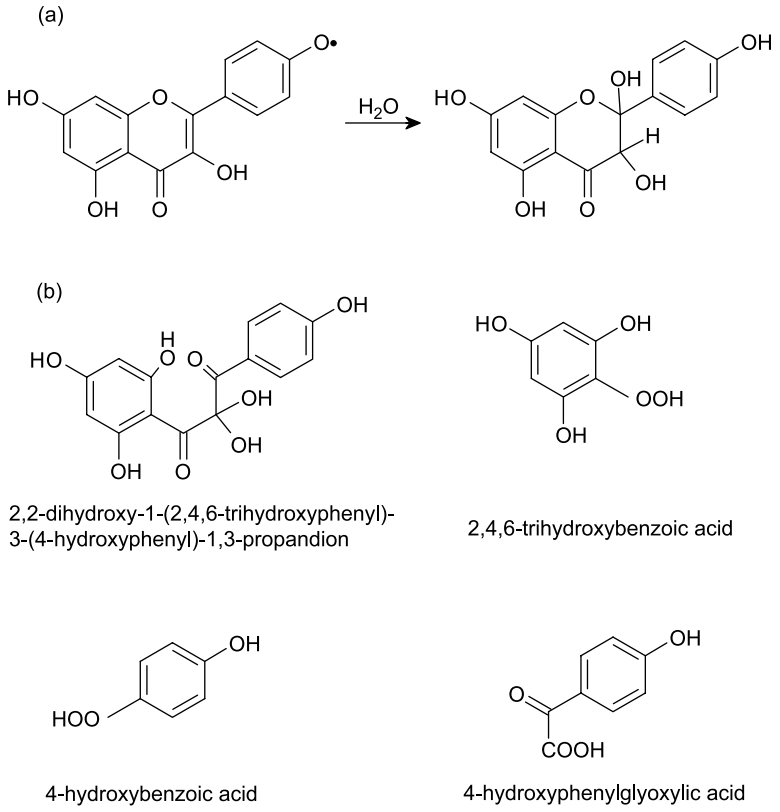

Figure 6. (a) Proposed first step of kaempferol oxidation; (b) detected breakdown products of kaempferol.

Takahama [19] detected two degradation products (2,4,6-trihydroxybenzoic acid and 4-hydroxyphenylglyoxylic acid; Figure 6b) from the oxidation of kaempferol by superoxide anion radicals, but neither had a structure that correlates with our EPR results. There are some reports about inhibitory effects of kaempferol on the enzyme XO [11,20,21], where it is supposed that OH-groups on carbon 5 and 7 block the position in the enzyme where xanthine should add. Since in our experiments the X/XO reaction had already commenced before addition of the flavonol, any inhibitory effect will have no consequence for the initial radical generation in the reaction with kaempferol.

Puppo [22] has reported that kaempferol can function as either an antioxidant or a prooxidant in the Fenton reaction depending on the experimental conditions. In the presence of Fe(III), EDTA and H_2O_2, kaempferol stimulates $^{\cdot}OH$-formation by helping the redox-cycling of iron, and presumably undergoes one-electron oxidation to form a phenoxyl radical, which breaks down as described in the autoxidation experiments. This is supported by the observation of an EPR signal from the *p*-benzosemiquinone radical. Puppo also stated that kaempferol can act as an $^{\cdot}OH$-radical scavenger. However, the reaction between kaempferol and $^{\cdot}OH$ radicals is likely to be less specific than oxidation by Fe(III) EDTA and a number of radical centres could be formed from the flavonol. Such radicals could be the source of the di- or polymeric structure that is necessary to explain the ^1H hyperfine splitting constants in the sextet EPR spectrum obtained with this system.

The spectrum of the first EPR signal reported by van Acker et al. [12] for the autoxidation of luteolin appeared identical to our spectrum (Figure 2a), but their reported hyperfine splitting constants produced a completely different spectrum on simulation using the Bruker Simfonia software. We suggest, therefore, that there is an error in the parameters reported in this paper. In addition, van Acker et al. [12] were not able to detect an EPR signal when using HRP and H_2O_2 at pH 7.4 as the oxidising agent. The difference between this result and that in the present paper may be a consequence of the different experimental setups used. In our case the flavonoid was the last component to be added to the solution, whereas van Acker et al. [12] included the flavonoid in the radical generating system. Recently, Huang et al. [23] have reported that HRP can be inactivated by phenoxyl radicals generated by reaction of H_2O_2 with phenols. If luteolin has a similar effect on the enzyme, this could explain the failure of van Acker et al. [12] to observe radical formation in their HRP/H_2O_2/luteolin system.

Conclusions

Although the structures of luteolin and kaempferol differ only in the position of one OH-group and the antioxidant behaviour of these two molecules is similar, the behaviour of these molecules under oxidative conditions are completely different. The catechol group of luteolin stabilizes the radical anion and hence prevents degradation. In contrast, the initial phenoxyl radical formed by oxidation of kaempferol is unstable, and fragments. Thus, whereas luteolin could be redox cycled, the biological activity of kaempferol is probably related to its degradation products.

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