Exploration of pro-oxidant and antioxidant activities of the flavonoid myricetin

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Objectives: Flavonoids are ubiquitous phenolic plant metabolites. Many of them are well known for their pro- and antioxidant properties. Myricetin has been reported to be either a potent antioxidant or a pro-oxidant depending on the conditions. The reaction conditions for the pro- and antioxidant activities were therefore investigated using variations of the deoxyribose degradation assay systems.

Methods: The deoxyribose degradation assay systems were conducted as follows; $\text{H}_2\text{O}_2/\text{Fe}^{\text{III}}$ /ascorbic acid, $\text{H}_2\text{O}_2/\text{Fe}^{\text{III}}$, Fe^{III} /ascorbic acid, and Fe^{III} . Each system was carried out in two variants, FeCl_3 (iron ions added as FeCl_3) and FeEDTA (iron added in complex with ethylenediaminetetraacetic acid).

Results: When ascorbic acid was present, myricetin showed antioxidant properties, especially when it occurred in complex with iron. In ascorbic acid-free systems, pro-oxidant activities prevailed, which were enhanced if iron was in complex with EDTA.

Discussion: Myricetin's antioxidant activity depends on both the reactive oxygen species (ROS) scavenging and iron ions chelation properties. The pro-oxidative properties are caused by reduction of molecular oxygen to ROS and iron(III) to iron(II). Myricetin is able to substitute for ascorbic acid albeit less efficiently.

Keywords: Flavonoids, Free radical scavengers, Hydroxyl radical, Iron chelating agents, Reactive oxygen species

Introduction

Flavonoids are widely occurring plant phenols. Their effects on human health have been described repeatedly,¹ but the actual benefit to the producing plant is still controversial, although various explanations have been offered.² One such advantage may be redox homeostasis regulation by scavenging or generation of reactive oxygen species (ROS).^{3,4} ROS are better known for causing cytotoxicity, but also may affect various cellular signal cascades and metabolic processes.⁵ ROS are required in specific low concentrations for normal cell functioning.^{3,5,6} Many plant secondary metabolites act as antioxidants and pro-oxidants and can affect ROS concentrations depending on the reaction conditions.^{3,7} In aromatic compounds such as flavonoids, antioxidant activities depend on the redox properties of the phenolic hydroxyl groups, especially on those of the catechol or pyrogallol moieties, which are easily oxidized.^{8,9} Reduction of molecular oxygen to superoxide anion radical and iron(III) to iron(II) ions can cause pro-oxidant activities.⁹ Many of these redox reactions, however, are

Figure 1 Structure of myricetin.

reversible, the specific reaction mixture and conditions resulting in complex equilibria.^{10,11}

Myricetin (Fig. 1) serves as a good example of the complexity of this phenomenon.¹² Controversial reports on myricetin's effects, both as an antioxidant and a pro-oxidant, stimulated us to evaluate its redox properties in the deoxyribose degradation assay, which was originally developed to assess test compound reactions with hydroxyl radicals ($\text{HO}^\bullet$) that are produced by the Fenton reaction:^{13,14}

It utilizes the oxidative degradation of 2-deoxy-D-ribose by hydroxyl radicals as the detection mechanism for antioxidative activity. The degradation

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products can be quantified photometrically after reacting with 2-thiobarbituric acid to thiobarbituric acid-reactive species (TBARS).¹¹

We performed the deoxyribose degradation assay in four systems:¹¹

1. $H_2O_2/Fe^{III}/ascorbic\ acid$: Hydroxyl radicals are generated by the Fenton reaction after reduction of iron(III) to iron(II) by ascorbic acid.^{13,14} This system shows possible interactions of the tested compound with the Fenton reaction and oxidative degradation of 2-deoxy-D-ribose.
2. H_2O_2/Fe^{III} : If ascorbic acid is omitted from the reaction mixture, the tested compound can start the Fenton reaction by reducing iron(III).¹⁵ This assay modification detects a possible replacement of ascorbic acid by the test compound in the reaction mixture. The reaction of the tested compound with iron(III) has to be kinetically more favorable than that of hydrogen peroxide.
3. $Fe^{III}/ascorbic\ acid$: Ascorbic acid reduces iron(III) to iron(II), which reacts further with molecular oxygen present in the reaction mixture by generating superoxide anion radicals.^{11,16} These dismutate to molecular oxygen and hydrogen peroxide. The superoxide anion radicals may also be reduced to hydrogen peroxide by one electron transfer from ascorbic acid or the tested compound. Iron(III) reduction and hydrogen peroxide formation start the Fenton reaction. This system explores possible interactions of the test compound with hydroxyl radical generation by auto-oxidation of ascorbic acid.
4. Fe^{III} : In the presence of iron(III) ions, the test compound may be oxidized and, in consequence, iron(III) may be reduced to iron(II) which then reduces molecular oxygen present in the reaction mixture to superoxide anion radical. The latter dismutates into molecular oxygen and hydrogen peroxide which starts the Fenton reaction.¹¹

To all these systems, iron(III) is added in two variants, either as $FeCl_3$ or in complex with ethylenediaminetetraacetic acid (EDTA). If iron ions are added as chloride, they can form a direct complex with the test compound¹³ facilitating studying of the test compound–iron complex. An already-formed iron(III)–EDTA complex, however, prevents complex formation of iron with other ligands and allows exploration of the chemistry of the test compound when it is not a ligand of a transition metal.¹⁵

Materials and methods

Chemicals

Hydrogen peroxide and 2-deoxy-D-ribose were obtained from Fluka (Buchs, Switzerland). All other chemicals used were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Water had Milli-Q quality.

Deoxyribose degradation assay

The various systems and variants of the assays follow procedures of which detailed reaction mechanisms are

described elsewhere.¹¹ In short, the assays comprise two systems: in one, H_2O_2 is added and, in the other, absent. Accordingly, since H_2O_2 an important reactant for the Fenton reaction was missing in the second system, additional time was needed to allow oxygen to diffuse into the reaction solution and to be subsequently reduced to ROS. Each system was performed in two sets (ascorbic acid present or absent); the latter set aimed to explore if myricetin can substitute for ascorbic acid. Furthermore, there are two variants: Iron was either added as $FeCl_3$ ($FeCl_3$ variant) or as Fe^{III} complex with EDTA ($FeEDTA$ variant). Myricetin was dissolved in a dilution series (2–500 μM) in an aqueous KH_2PO_4/KOH buffer solution (50 mM, pH 7.4); to 125 μl of this solution, 25 μl of a 10.4 mM 2-deoxy-D-ribose solution in the same buffer system and 50 μl of aqueous $FeCl_3$ solution (50 μM) were added. In one series, the latter 50 μl contained 52 μM EDTA dissolved in buffer, which was premixed with the aqueous $FeCl_3$ solution (1:1 v/v). In the other series, the EDTA volume was replaced by buffer. Further, 25 μl 10.0 mM aqueous solution of H_2O_2 and 25 μl of 1.0 mM ascorbic acid in the buffer were added to start the Fenton reaction in the $H_2O_2/Fe^{III}/ascorbic\ acid$ reaction mixture. In the other deoxyribose degradation assays systems, H_2O_2 or ascorbic acid was replaced by the same volume of water or buffer, respectively. TBARS were determined photometrically at 532 nm after reaction with thiobarbituric acid and subsequent extraction of the pink pigment with 1-butanol. The positive control was the $H_2O_2/Fe^{III}/ascorbic\ acid$ reaction mixture lacking the test compound (100% TBARS in all systems and variants, determined in each series of the experiments to facilitate result comparability). Blanks contained the full reaction mixtures except for 2-deoxy-D-ribose and were determined in each replicated experiment. Assays were performed in triplicates. The systems containing H_2O_2 were evaluated after 1 hour. The systems without H_2O_2 were evaluated after 16 hours. The temperature during incubation was 27°C.

Statistical analysis

Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, MD, USA) was used to perform analyses of variance (ANOVA) with 95% Duncan's multiple range test. The dose–response curves were calculated using the Brain–Cousens model,¹⁷ and performed with R¹⁸ using the package *drc*.¹⁹

Results

$H_2O_2/Fe^{III}/ascorbic\ acid$ system

Myricetin showed antioxidant effects (Fig. 2A). This effect was stronger in the $FeCl_3$ than in the $FeEDTA$ variant. Within the tested concentration range, $FeCl_3$ followed a full sigmoidal dose–response curve

Figure 2 Effects of myricetin on TBARS formation in various systems and variants of the deoxyribose degradation assay; 100% TBARS represents mean formation in the $FeCl_3$ or $FeEDTA$ variant of the classical ascorbic acid- H_2O_2 system; bars are mean $\pm$ SD; $N=3$, letters indicate different levels of significance between effects (95% Duncan's multiple range test).

(Fig. 3A) while $FeEDTA$ only showed a portion of this response (Fig. 3B). The antioxidant effects became more pronounced by increasing the ratio myricetin:Fe.

H_2O_2/Fe^{III} system

Myricetin was able to replace ascorbic acid in the reaction mixture and start the Fenton reaction in both variants (Fig. 2B). The pro-oxidant effect, however, changed according to the myricetin concentration and resulted in a non-linear U-shaped dose-response curve. This was visible in both variants and again less pronounced in the $FeEDTA$ variant (Figs 3C and D).

Fe^{III} /ascorbic acid system (ascorbic acid autoxidation)

In the $FeCl_3$ variant, myricetin showed antioxidant effects increasing with its concentration (Fig. 2C). In the $FeEDTA$ variant, it had pro-oxidant activity in lower concentrations and antioxidant effects in higher concentrations. The dose-response curves reflected the contrasting activities of the two variants (Figs 3E and F); an increasing myricetin:Fe ratio further stimulated antioxidant activity if iron was in complex with myricetin. If it was in complex with EDTA, the antioxidative activity was less pronounced.

Fe^{III} system (myricetin autoxidation)

In both variants of the assay, the pro-oxidant effect was clearly visible (Fig. 2D). The highest TBARS concentrations were found in the concentration range of 16–63 μM in the $FeCl_3$ variant and 31–250 μM in the $FeEDTA$ variant. Higher concentrations, however, decreased the pro-oxidative effect. In both variants, myricetin caused a U-shaped dose-response effect, which was more pronounced in the $FeEDTA$ variant (Figs 3G and H).

Discussion

All proposed reactions are hypothetical, representing only a portion of the possible ones, which could explain myricetin's anti oxidant and pro-oxidant activities in different chemical environments. Besides ROS scavenging, myricetin's iron chelation ability also affects its antioxidant efficacy – otherwise, the dose-response curves in both iron variants would be identical. This reaction behavior compares with the structurally, closely related flavonoid quercetin, which has one hydroxyl group less on ring B than myricetin,¹¹ confirming the notion that flavonoid pyrogallol or catechol moieties are oxidized first to semiquinones or quinones⁸ (Fig. 4). Flavonoids also slow the Fenton reaction by chelating iron ions, which affects the redox potentials of both the ligand

Figure 3 Dose-response curves of TBARS formation depending on the myricetin:Fe ratio in various systems and variants of the deoxyribose degradation assay; 100% TBARS represents mean formation in the FeCl_3 or FeEDTA variant of the classical ascorbic acid- H_2O_2 system; X-axis has logarithmic scaling but values are not transformed.

and the central atom.^{20–23} Iron-flavonoid complexes are more efficient antioxidant agents than the free flavonoids.²² Myricetin-iron(II) complexes have been shown to be stable and to compare with the well-known chelation agent desferoxamine at a neutral pH.^{20,24} Our experiments illustrate that iron-myricetin complexes are less efficient catalysts of the Fenton

Figure 4 Redox reactions of myricetin.

reaction than iron-EDTA or iron-ascorbic acid complexes. Consequently, we can show that iron complex formation represents a substantial component of myricetin's antioxidative activity.

In the absence of ascorbic acid (Figs 2B, D, 3C, D, G, and H), paradoxically, myricetin is a pro-oxidant. This suggests that myricetin is more or less able to substitute for ascorbic acid and reduce iron, molecular oxygen and ROS, albeit less efficiently than ascorbic acid itself (see Fig. 4). Our observations concur with previous literature reports.^{25,26} Myricetin reduced iron(III) to iron(II) in both variants of the assay system, FeCl_3 and FeEDTA , starting the Fenton reaction:

The myricetin semiquinone-iron(II) complex can reduce hydrogen peroxide to hydroxyl radical (Fig. 2B). If iron is in complex with EDTA, myricetin reduces the complex (Fig. 3D). In higher concentration, however, scavenging effects start to prevail due to the larger numbers of myricetin molecules present in the solution. These equilibrium dynamics contribute to a U-shaped dose-response effect (Figs 3C and D).

The pyrogallol structure of the ring B strongly adds to the pro-oxidant activity of myricetin; the related

catechol flavonoid quercetin was more or less inactive in the identical assay.¹¹ Min and Eber reported that myricetin increased calf thymus DNA nucleic base oxidation product concentrations in a hydrogen peroxide/iron(II) system more than quercetin.²⁷ These authors interpreted this phenomenon by suggesting that myricetin increases hydrogen peroxide concentration by autoxidation. In contrast, we show here that myricetin's pro-oxidant effect can also be affected by iron(II)/iron(III) redox cycling, probably more than by direct reduction of molecular oxygen. The kinetics of the iron(III) reduction is crucial for Fenton-type reactions.^{28,29} In complex with myricetin (or other structurally similar flavonoids), the iron(III) reduction is slowed at a neutral or weakly alkaline pH. Subsequent depletion of iron(II) slows the Fenton reaction.²⁰ Our results, which were obtained in specifically developed systems and variants of the deoxyribose degradation assay that aim at exploring the catalytic properties of transition metals on the redox chemistry of test compounds, illustrate that the reduction of iron(III) occurred in complexes with both ligands, EDTA and myricetin. With higher myricetin concentrations, however, the ROS scavenging activity of the pyrogallol moiety again starts to prevail, which inhibits 2-deoxy-D-ribose degradation.

Ascorbic acid is a common plant metabolite and renowned for its antioxidant properties aimed at maintaining the redox homeostasis in the cell.³⁰ Conversely, its reducing properties are utilized in the deoxyribose degradation assay to generate hydroxyl radicals from hydrogen peroxide; iron(II) is an important catalyst. The Fe^{III}/ascorbic acid system (Fig. 2C) indicates that, if iron is complexed by the strong ligand EDTA, less hydroxyl radicals are formed than if iron is in complex with ascorbic acid. This further illustrates that ligand quality can affect the redox potential of the central iron atoms in a complex.^{21,31} Myricetin probably removes the central atom from the iron-ascorbic acid complex, thus decreasing hydroxyl radical formation by changing the redox potential of iron in the new complex as well as by scavenging ROS; this may explain the six-fold reduction of TBARS levels already at a low-concentration range (Figs 3E and F). While pro-oxidant effects of myricetin, in the presence of ascorbic acid and EDTA, have already been documented,²⁵ the non-linearity of this reaction is a novel finding.

The scenario, however, offers even more complexity: Myricetin can reduce iron(III), not only in complex with EDTA, but, in higher concentrations, also in complex with other myricetin molecules (Fe^{III} system in Fig. 2D). This phenomenon is even better demonstrated in Figs 3G and H. A higher ratio myricetin:Fe increased TBARS production but in higher concentration myricetin's scavenging properties

again became more prominent. In both variants of the Fe^{III} system lacking hydrogen peroxide, ROS were generated following myricetin's autoxidation. The electrons were transferred to molecular oxygen giving rise to superoxide anion radicals. Dismutation then led to hydrogen peroxide. In contrast to quercetin, myricetin showed pro-oxidant effects with both Fe^{III} variants. The former induced TBARS production only in the FeEDTA variant, and no U-shaped effect was visible at higher concentrations up to the solubility limit.¹¹ The pyrogallol arrangement of ring B is probably crucial for iron central atom reduction in the flavonoid complex in a weakly alkaline pH. An electron paramagnetic resonance study²⁰ identified a semiquinone signal of the autoxidized myricetin after iron ion addition at a weakly alkaline pH.²⁰ In agreement with our results, two studies have reported autoxidation of myricetin in the presence of iron ions and subsequent ROS production.^{25,32} ROS were formed via molecular oxygen reduction to superoxide anion radical.³³

Conclusion

The systems and variants of the deoxyribose degradation assay allow substantial insights into the autoxidation dynamics of the pyrogallol moiety of myricetin. It increases the reducing power of the flavonoid, which also can result in pro-oxidant activity. Firstly, liganded iron(III) is reduced to iron(II). The autoxidation of iron(II) transfers electrons to molecular oxygen, resulting in the formation of superoxide anion radicals and other ROS. Myricetin's pro- and/or antioxidant effects depend on both the concentration of the reactants and reaction conditions. The final outcome is determined by many reversible and simultaneous reactions as evidenced by the U-shaped dose-response curves.

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