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ANTIOXIDANT ACTIVITY OF FLAVONOIDS AND REACTIVITY WITH PEROXY RADICAL

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Key Word Index—Flavonoids; antioxidant; polyunsaturated fatty acids; hydroperoxide; peroxy radical.

Abstract—The autoxidation of linoleic acid and methyl linolenate is inhibited by flavonoids. The antioxidant efficiency of these flavonoids increases with their concentration and in the order fustin < catechin < quercetin < rutin = luteolin < kaempferol < morin for linoleic acid and rutin < catechin < morin = kaempferol for methyl linolenate. Flavonoids are more effective on linoleic acid than on methyl linolenate. The antioxidant activity of flavonoids is related to an inhibition of the formation of *trans,trans* hydroperoxide isomers of linoleic acid. This inhibition exhibited the great H-atom donating ability of flavonoids to peroxy radical, thus terminating the chain radical reaction.

INTRODUCTION

The flavonoids comprise a large family of therapeutic agents which possess vitamin P properties. They protect against vascular disorders by decreasing the permeability and fragility of capillaries. The mechanism of their action, could be related to their antioxidant function [1] since vascular disorders may be caused by oxidative damage of cell membranes. Polyunsaturated fatty acids, present in cell membranes, are easily oxidized both by enzymatic and autoxidative peroxidation via free radical chain reactions [2, 3].

Initiation (I) $RH \rightarrow R^{\cdot}$

Propagation (II) $R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$

Termination (III) $ROO^{\cdot} + ROO^{\cdot} \rightarrow$ inert product
$R^{\cdot} + R^{\cdot} \rightarrow$ inert product
$ROO^{\cdot} + R^{\cdot} \rightarrow$ inert product.

Initiation of lipid peroxidation (I) can be induced by free radicals ($O_2^{\cdot-}$, $^{\cdot}OH$) and singlet oxygen ($^1O_2$) produced in biological systems [4–8]. Thus, lipid peroxidation may be prevented at the initiation step by singlet oxygen quenchers or by free radical scavengers. Furthermore, the propagation chain reaction (II) can be broken by peroxy-radical scavengers such as phenolic compounds (AOH) [9].

$$ROO^{\cdot} + AOH \rightarrow ROOH + AO^{\cdot}$$

It has been shown that lipid peroxidation can be inhibited by flavonoids acting as strong $O_2^{\cdot-}$ scavengers [10] and $^1O_2$ quenchers [1]. It has also been proposed that flavonoids react with peroxy radicals involving termination of radical chain reactions. This latter mechanism was inferred from the oxidation of quercetin and rutin by lauroyl peroxide radicals [11].

The purpose of this work is to point out the reactivity of some flavonoids with peroxy radicals formed during the autoxidation of poly-unsaturated fatty acids as a consequence of the distribution of hydroperoxide isomers of fatty acids.

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RESULTS AND DISCUSSION

Autoxidation rate of fatty acids with flavonoids

The addition of flavonoids slows down the level of conjugated dienes ($A_{234\,nm}$) formed during the autoxidation of both linoleic acid and methyl linolenate in an aqueous media (Figs 1 and 2). The antioxidant efficiency of these flavonoids depends on: (a) their concentration. An increase of the concentration from 1 to 100 μg/ml, increases the antioxidant efficiency of flavonoids (Fig. 3). (b) The degree of unsaturation of the fatty acid. An increase in the unsaturation results in a decrease of the antioxidant efficiency. Flavonoids, at a given concentration, are more effective as antioxidants on linoleic acid than on methyl linolenate (Fig. 1 compared to Fig. 2). (c) The flavonoid itself. The antioxidant efficiency increases in the order fustin < catechin < quercetin < rutin = luteolin < kaempferol < morin for linoleic acid and rutin < catechin < morin = kaempferol for methyl linolenate.

Distribution of linoleic acid hydroperoxides with flavonoids

As previously described [13–15] the autoxidation of linoleic acid leads to the formation of four hydroperoxide isomers: 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic; 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic; 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic; 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acids. At 10 days of autoxidation, *ca* 25% of each hydroperoxide isomer are present in aqueous samples [16]. The addition of phenolic compounds such as flavonoids partially inhibited the formation of 13 *trans,trans* and 9 *trans,trans* isomers. This inhibition increases in the order: fustin < catechin < quercetin < rutin = luteolin

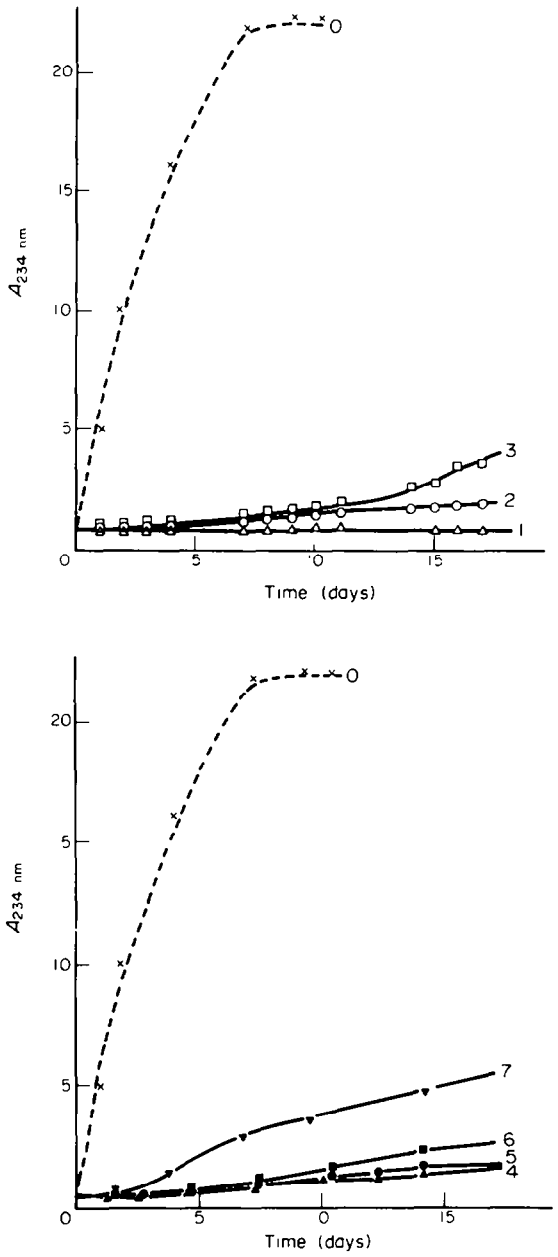


Fig. 1. Level of conjugated dienes from autoxidized linoleic acid with and without flavonoids. A, Linoleic acid x--x, 0 alone; 1 △—△, with morin (10 μg/ml); 2 ○—○, with rutin (10 μg/ml); 3 □—□, with catechin (10 μg/ml). B, Linoleic acid x--x 0 alone; 4 Δ—Δ, with kaempferol (10 μg/ml); 5 ●—●, with luteolin (10 μg/ml); 6 ■—■, with quercetin (10 μg/ml); 7 ▼—▼, with fustin (10 μg/ml).

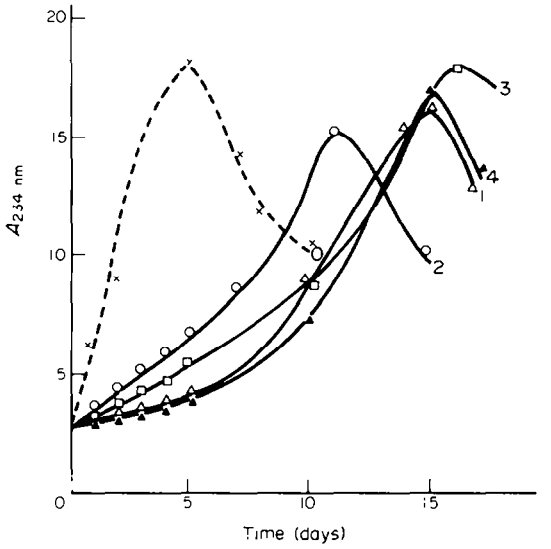


Fig. 2. Level of conjugated dienes from autoxidized methyl linolenate with and without flavonoids. Methyl linolenate x—x, 0 alone; 1 △—△, with morin (10 μg/ml); 2 ○—○, with rutin (10 μg/ml); 3 □—□, with catechin (10 μg/ml); 4 ▲—▲, with kaempferol (10 μg/ml).

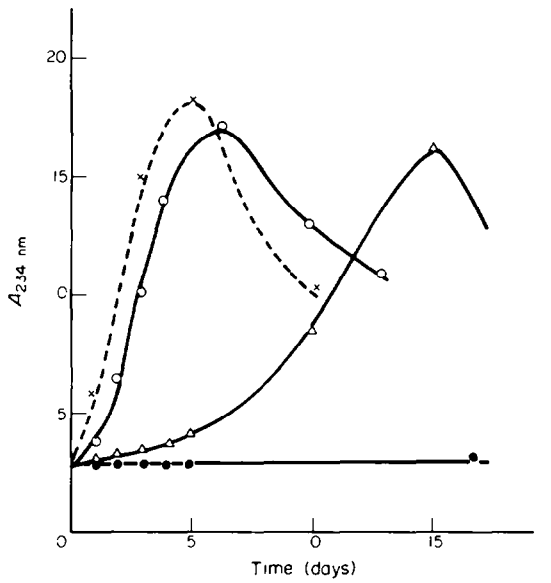
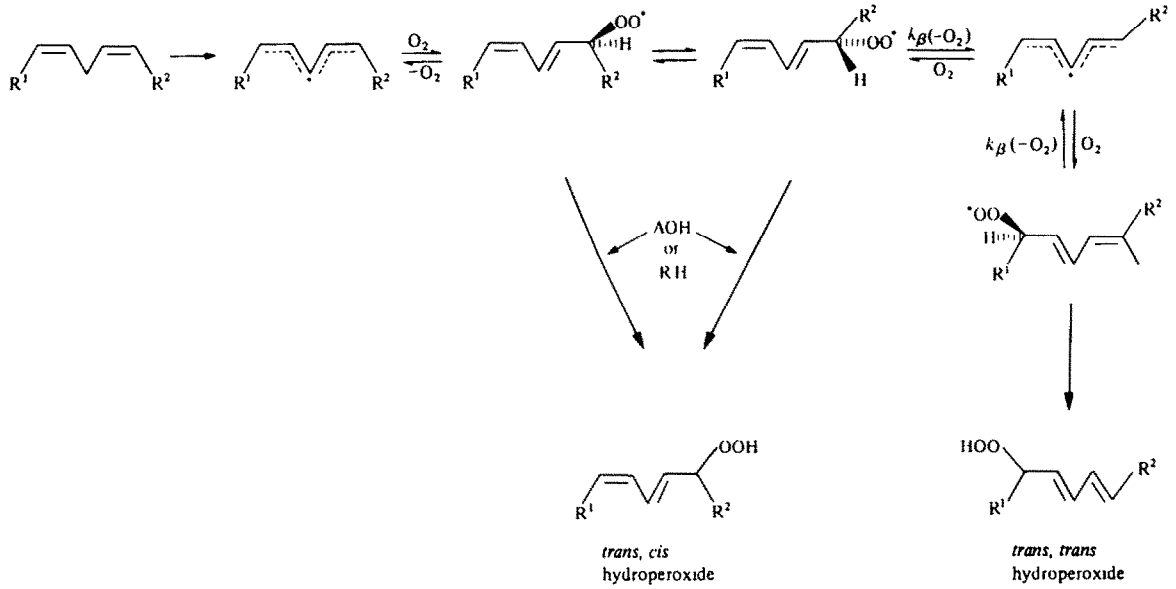


Fig. 3. Effect of morin concentration on the level of conjugated dienes from autoxidized methyl linolenate. Methyl linolenate x--x alone; ○—○, with morin (1 μg/ml); △—△, with morin (10 μg/ml); ●—●, with morin (100 μg/ml).

< kaempferol < morin. The antioxidative activity of flavonoids is related to an inhibition of the formation of *trans,trans* hydroperoxide isomers. The same inhibition was observed with α-tocopherol at high concentration [16–18].

Weenen and Porter [18] have proposed a mechanism which accounts for the formation of *cis,trans* and *trans,trans* hydroperoxide isomers of linoleic acid. In this mechanism, H-atom abstraction from fatty acid (RH) and oxygen entrapment gives a peroxy radical (ROO·). Then the crucial competition that determines the *trans,cis/trans,trans* ratio is β scission of peroxy radical which ultimately leads to a *trans,trans* product. Competing with the scission of peroxy radical is H-atom abstraction from the fatty acid that leads to hydroperoxide. If H-atom donors such as phenolic compounds



Scheme 1. Formation of *cis,trans* and *trans,trans* hydroperoxide isomers with and without flavonoids (AOH).

are added to reaction mixture, the formation of *trans,trans* isomer is thus decreased (Scheme 1).

The inhibition of the formation of *trans,trans* isomers by flavonoids showed that these compounds act as H-atom donors to the peroxy radical, thus inhibiting the autoxidation of fatty acids by chain radical termination.

EXPERIMENTAL

Reagents and chemicals. Methyl linolenate (≥ 99% pure) rutin (95% pure), morin, (+)-catechin, kaempferol, were supplied by Sigma; quercetin, luteolin from Sarsyntex (France) and fustin from Fluka. Linoleic acid (≥ 99% pure) was purchased from Koch Light, Tween 20 from Merck, Na_2Pi, MeOH, $CHCl_3$ and HOAc from Prolabo (France) and *n*-heptane 'chromasol' from S.D.S.

Autoxidation of fatty acids with phenolic compounds. Polyunsaturated fatty acid (linoleic acid or methyl linolenate) and phenolic compounds were dispersed with 0.5% Tween 20 in a Na phosphate buffer soln at pH 7 [12]. In all the samples, the initial concn of fatty acid was 2.5×10^{-3} M whereas each phenolic compound was initially present at three concns, respectively 1, 10 and 100 μg/ml. The samples were left in the dark and under air at room temp. Controls without fatty acid were placed under the same conditions.

Measure of the autoxidation rate of polyunsaturated fatty acids. The autoxidation of polyunsaturated fatty acids was accompanied in the early stage by the formation of hydroperoxides with a conjugated diene system which exhibited an absorption at 234–235 nm. Measurement of an increase of this absorption was achieved by a Pye Unicam SP 8-400 Spectrophotometer. Furthermore, the unoxidized fatty acid was determined by GC as previously described [12].

Determination of linoleic acid hydroperoxides. The hydroperoxide isomers of linoleic acid were extracted from the sample at 10 days of autoxidation. Then they were separated by HPLC as previously described [13]. HPLC was achieved on a stainless steel column (20 cm × 0.47 cm) of Spherisorb Si 60 (particle size 3–4 μm). The solvent was composed of *n*-heptane and HOAc (40:1) and the flow rate was 2 ml/min. The UV detection was at 240 nm.

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