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ORIGINAL PAPER

On the difference in decomposition of taxifolin and luteolin vs. fisetin and quercetin in aqueous media

Romana Sokolová[1] · Šárka Ramešová[1] · Jana Kocábová[1] · Viliam Kolivoška[1] · Ilaria Degano[2] · Emanuela Pitzalis[3]

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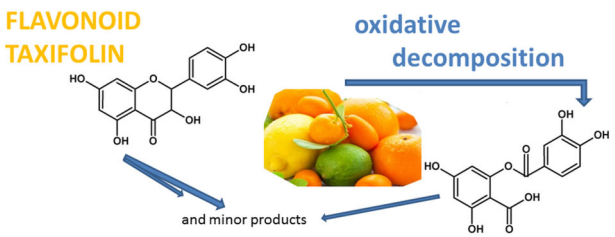
Abstract The decomposition of flavonols quercetin and fisetin, flavone luteolin and flavanone taxifolin was studied in slightly alkaline solution under ambient conditions. The study was based on spectrophotometry and high-pressure liquid chromatography. Products formed by atmospheric oxygen oxidation and hydrolysis were identified by HPLC–DAD and HPLC–ESI-MS/MS. Only small differences in the chemical structure of flavonoids resulted in extremely variable oxidation pathways and products. Oxidation of flavonols led to the formation of both a benzofuranone derivative and several open structures. On the contrary, the benzofuranone derivative was not found as a product of taxifolin and luteolin oxidative decomposition. These compounds were oxidized to their hydroxylated derivatives and typical open structures. Quercetin was not identified as a possible oxidation product of taxifolin.

Graphical Abstract



✉ Romana Sokolová
sokolova@jh-inst.cas.cz

[1] J. Heyrovský Institute of Physical Chemistry of ASCR, v.v.i., Dolejškova 3, 18223 Prague, Czech Republic

[2] Department of Chemistry and Industrial Chemistry, University of Pisa, Via Moruzzi 13, 56124 Pisa, Italy

[3] Institute of Chemistry of Organometallic Compounds, CNR, Via G. Moruzzi, 1, 56124 Pisa, Italy

Keywords Oxidation mechanism · Fisetin · Luteolin · Taxifolin · Quercetin

Introduction

The redox properties of bioactive compounds and drugs can give information about their role in metabolic processes or their pharmacological activity [1–3]. The bioactivity is also connected to the iron chelation ability and the affinity of catechols to compete with iron binding proteins [4, 5]. Electrochemical methods are frequently employed as a part of biomimetic modelling of reductive or oxidative drug metabolism [6, 7].

Flavonoid compounds are widely distributed in nature, in fruits, vegetables, and leaves [8, 9]. Presently, these compounds are extensively studied in aqueous solution because of their pharmacological importance. They have antioxidative, anticarcinogenic, anti-inflammatory, and antiviral properties [10–12]. The chemical class of flavonoids includes flavonols, flavones, isoflavonoids, anthocyanins, flavanones, etc. [13]. Taxifolin (**1**, 3,5,7,3′,4′-pentahydroxyflavanone; Fig. 1), which occurs in citrus fruits, belongs to the family of flavanones [13]. It is characterized by a relatively strong antioxidant activity due to the substitution with two OH groups at the position 3′ and 4′ of the B-ring. Its molecule does not contain double bond between carbon atoms C2 and C3, and thus its antioxidant activity is half of that of quercetin (**2**, 2,3,3′,4′,5,7-pentahydroxyflavone; Fig. 1) [14]. Despite that, its protective effect against cerebral ischemic reperfusion injury and leukocyte activation was reported [15, 16]. Luteolin (**3**, 3′,4′,5,7-tetrahydroxyflavone; Fig. 1) is widely present in plants, vegetables as artichoke, basil, celery, and parsley [8]. It is a flavone, whose

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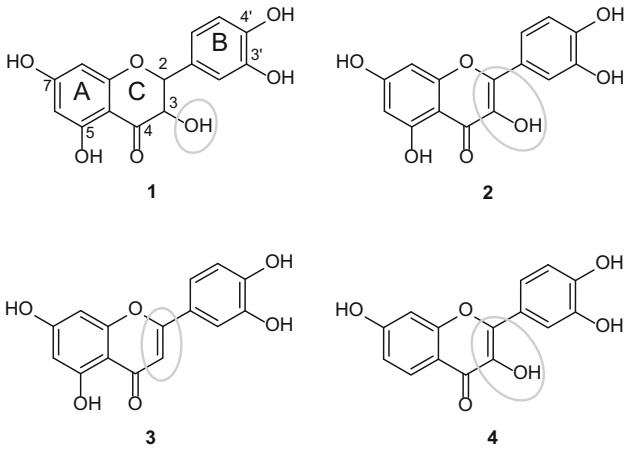


Fig. 1 Chemical structure of taxifolin (**1**), quercetin (**2**), luteolin (**3**), and fisetin (**4**)

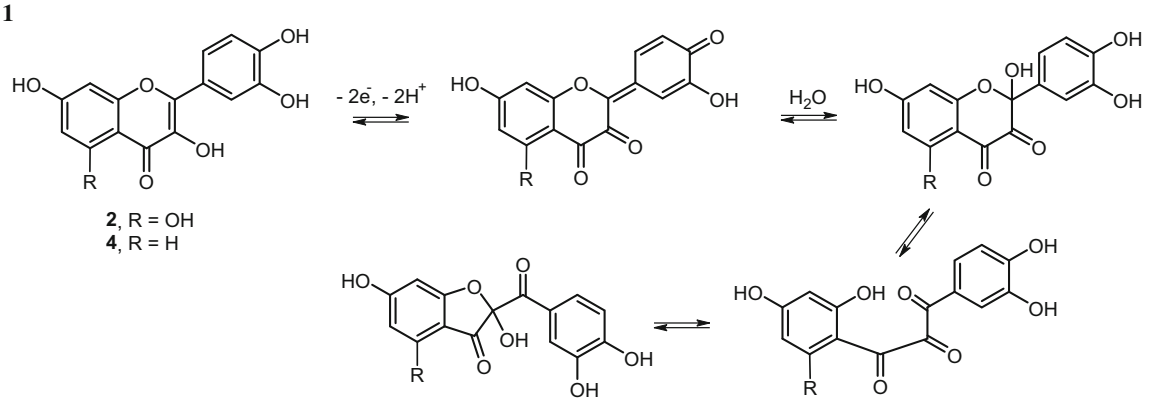
chemical structure differs from quercetin by the absence of C3–OH hydroxyl group. It exhibits antioxidant, anti-inflammatory, and anti-allergic activity [17, 18]. Fisetin (**4**, 2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one; Fig. 1), another bioactive flavonoid present in nature, has promising antioxidant, anticarcinogenic, and antibacterial properties [11, 19].

The oxidation mechanism of the flavonol structure was extensively studied in literature for quercetin (**2**). The large variety of oxidation products of quercetin described in the literature by several authors is related to the instability of the primary formed oxidation products [20]. The oxidation of flavonols in buffered solutions under inert atmosphere of argon showed that both the oxidation potential and the number of electrons and protons involved in the oxidation strongly depend on the relative amount of dissociation forms of flavonol in solution [21–23]. In the case of quercetin, subsequent hydroxylation and the formation of 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3-(2*H*)-one take place (Scheme 1). This product was also confirmed as the only oxidation product of quercetin formed under inert atmosphere in non-aqueous solution and in the cavity of β-cyclodextrin [24, 25]. This benzofuranone derivative was isolated as the oxidation product of quercetin and identified it by ^{1}H NMR and ^{13}C NMR [26], it was also generated from quercetin by the peroxyl radical generator 2,2′-azobisisobutyronitrile [27]. Consistently, 2,4-dihydroxy-2-(4-hydroxy-3-methoxybenzoyl)-6-methoxybenzofuran-3(2*H*)-one was identified as the only oxidation product of the methoxylated flavonol rhamnazin under inert atmosphere [23]. Finally, the corresponding benzofuranone derivative was confirmed after the oxidation of fisetin (**4**) both in solution exposed to the air and degassed with argon [22].

Interestingly, the electrochemical oxidation of taxifolin [28] and luteolin [29] under inert atmosphere of argon did not show the formation of a benzofuranone derivative as it was in the case of quercetin and fisetin [21, 22, 30]. In the case of taxifolin, the redox properties are affected by its specific structure, which does not allow the electron delocalization through the whole structure [28]. Although this effect of delocalization of electrons is allowed in the case of luteolin, the absence of formation of the benzofuranone derivative of luteolin suggests that the highly reactive C3–OH hydroxyl group has a significant contribution to the whole oxidative process [31, 32]. The electrochemical oxidation of both, luteolin and taxifolin, yielded to the formation of related quinone derivatives and its subsequent hydroxylation to its hydroxyl derivatives [28, 29].

Under ambient conditions, flavonols quercetin and fisetin were found to decompose fast [21, 22]. This phenomenon made difficulties in the analytical determination of oxidation products formed by oxidative electrolysis under inert atmosphere. The purpose of this work is to investigate the mechanism of oxidative decomposition of flavanone taxifolin and flavone luteolin in aqueous solution under ambient conditions. Their behaviour will be

Scheme 1



compared with the decomposition of flavonols quercetin and fisetin.

Result and discussion

Flavanone taxifolin

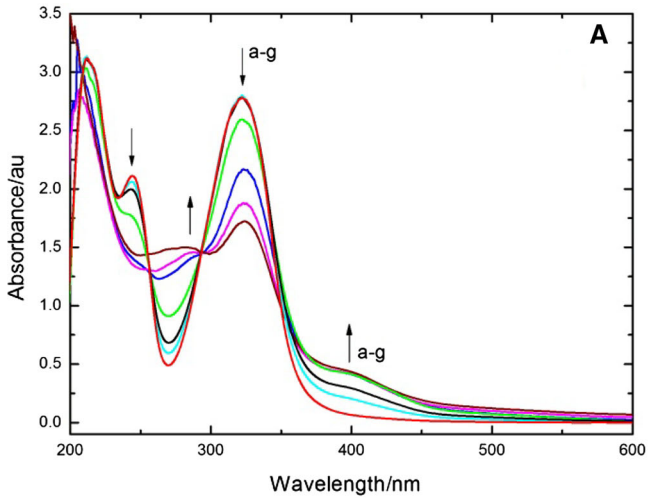
The oxidative decomposition of taxifolin (**1**) was first studied by means of UV–Vis spectrophotometry. Figure 2a shows the changes of the absorption spectra of **1** depending on the time of exposure to the air oxygen. The absorption spectrum of **1** under argon atmosphere is characterized by bands with absorption maxima at 213, 244, and 321 nm. During exposure to atmospheric oxygen the intensity of absorption bands at 244 and 321 nm decrease. At the same time the absorption bands at 286 and 400 nm increase. In 20 min the absorbance at 400 nm reaches the limiting value. It is obvious that a significant change of the absorption spectrum of **1** occurs at much longer times than in the case of luteolin (**2**) (Fig. 2b).

The solution of taxifolin prepared under inert atmosphere of argon was injected in the HPLC system. The chromatogram is shown in Fig. 3a. Afterwards, the vial was opened and the solution was exposed to the air and shaken. Chromatograms registered at different times of exposure to air oxygen are reported in Fig. 3b and c. Several peaks labelled as T1–T9 due to the oxidation products of **1** occurred in the chromatograms. To compare the oxidation products with the already known oxidation products of quercetin (**2**) [21, 33], the chromatogram of quercetin standard **2** was measured under the same conditions. It was verified that none of the oxidation products of **1** corresponded to compound **2** (compare Fig. 3b, c to d).

Oxidation products of compounds taxifolin and quercetin are summarized in Table 1. They were identified by comparison of their absorption spectra obtained from HPLC–DAD and confirmed by means of HPLC–ESI-MS/MS analysis. The oxidation products of quercetin decomposition were additionally identified by GC–MS analysis after derivatization with silylating agent. Table 1 includes the *m/z* values obtained for oxidation products of all studied compounds. Scheme 2 shows chemical structures of taxifolin oxidation products.

Open structures

The formation of so-called 'open structures' was expected (the compound 2-(3,4-dihydroxybenzyloxy)-4,6-dihydroxybenzoic acid is here called 'open structure—acid' for clarity reasons and applies to all the analogous products formed for all the studied compounds). Such products were found for **2** by Osman et al. [34]. Other 'open structures' were identified as the oxidation products of flavonols fisetin (**4**) [22] (compound 2-(3,4-dihydroxybenzoyloxy)-4-hydroxybenzaldehyde is here called 'open structure—aldehyde') and rhamnazin [23] (compound 2-hydroxy-6-(4-hydroxy-3-methoxybenzoyloxy)-4-methoxyphenyl-2-oxoacetic acid is here called 'open structure—oxoacetic acid'). Figure 4 shows the chemical structures of all open structures types found by oxidation of flavonols, flavones, or flavanones in our studies. These compounds are not stable, and readily undergo hydrolysis and low molecular weight hydroxyl compounds are formed (see Table 1).



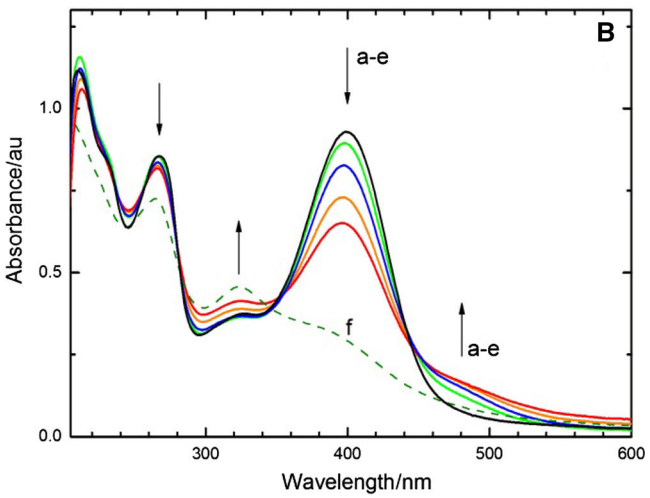


Fig. 2 **a** Shows the absorption spectra of 2.4×10^{-4} M taxifolin in 3.6×10^{-3} M KOH during the exposure to the air oxygen for *a* 0, *b* 600 s, *c* 900 s, *d* 1200 s, *e* 19 h, *f* 40 h, *g* 7 days. **b** Shows the absorption spectra of 3.5×10^{-5} M luteolin in 3.6×10^{-3} M KOH during exposure to atmospheric oxygen for *a* 0, *b* 740 s, *c* 1240 s, *d* 1920 s, *e* 2700 s, *f* 24 h

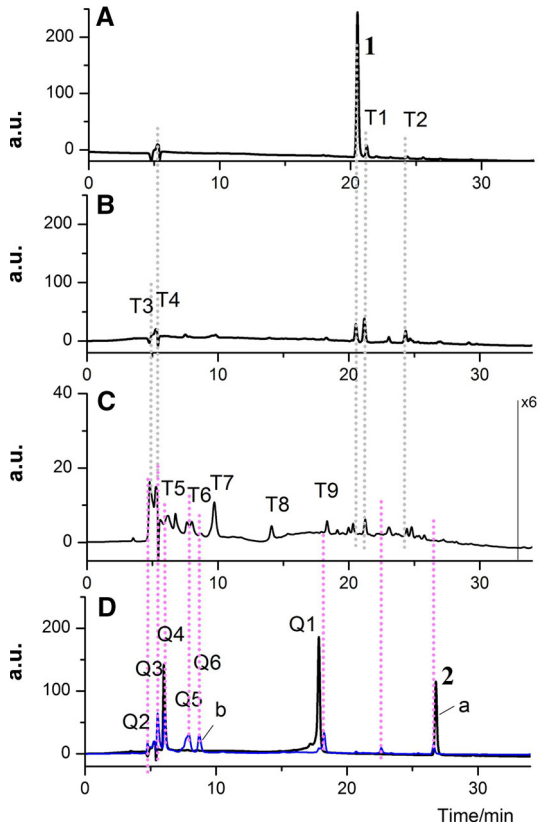


Fig. 3 HPLC chromatogram of taxifolin in 0.1 M KCl and 5.4×10^{-3} M KOH recorded after exposure to atmospheric oxygen for different times: **a** 0 min, **b** 60 min, **c** 28 h. **d** Shows chromatogram of solution of **2** *a* before and *b* after oxidation by oxygen. Detector set at 280 nm

Flavone luteolin

The absorption spectrum of luteolin (**3**) in 3.6×10^{-3} M KOH is characterized by four bands with absorption maxima at 212, 266, 324, and 397 nm (Fig. 2b). The absorption bands at 266 and 397 nm decrease during the exposure of solution to oxygen. A new absorption band at 480 nm occurs, which is due to the formation of hydroxylated luteolin [29, 35]. Oxidation products found after the oxidation of flavone luteolin are summarized in Table 1 and Scheme 3.

Flavonol fisetin

Oxidation of fisetin (**4**) under ambient conditions leads to the formation of 2-(3,4-dihydroxybenzoyl)-2,6-dihydroxybenzofuran-3(2*H*)-one (**F2**). This benzofuranone structure was found to be typical oxidation product of flavonols [21–23, 30]. Other oxidation pathways yield 2-(3,4-dihydroxybenzoyloxy)-4-hydroxybenzaldehyde (**F1**, open structure—aldehyde) and 2-(3,4-dihydroxybenzoyloxy)-4-hydroxybenzoic acid (**F3**, open structure—acid) as shown in Fig. 5 and Scheme 4. The oxidation product **F3** elutes at very close retention time to benzofuranone derivative **F2**. The area of peak **F3** in the chromatograms increased with time when the solution was exposed to oxygen (Fig. 5b). Other peaks belonging to low molecular weight hydroxyl compounds were identified, as summarized in Table 1. Amongst the investigated flavonols, only in the oxidized solutions of fisetin a benzofuranone derivative could be distinguished from the 'open structure—acid'.

Conclusion

The present complex study was focused on the relationship between the oxidation properties of several flavonoid compounds in air, and their chemical structure. The difference in their chemical structures is often very small, as the absence of one C3–OH hydroxy group or the absence of C2–C3 double bond; but a significant influence on electron transfer properties was assessed. Our recent works showed that flavonol compounds, containing both C2–C3 double bond and C3–OH hydroxy group in their chemical structure formed a benzofuranone derivative during their oxidation [21, 22]. In this paper, we prove that those compounds which do not fulfil both these conditions do not yield a benzofuranone derivative during their oxidation, even in the presence of air oxygen. This feature was assessed for flavanone taxifolin (the absence of double bond in comparison to flavonol quercetin) and flavone luteolin (the absence of C3–OH hydroxy group). For these compounds, two typical oxidation pathways were determined. The first pathway led through a quinone intermediate to a hydroxylated compound formed by nucleophilic addition of water. The second process was the formation of open structures and their subsequent decomposition to low molecular weight hydroxy compounds. Similar open structures were identified also in the case of flavonols quercetin and fisetin as side oxidation products.

Experimental

Fisetin, quercetin, taxifolin, and luteolin were purchased from Sigma Aldrich. Chemicals used for preparation of Britton–Robinson buffers of pH range 4.4–11.2 (0.04 mol dm^{-3} stock solutions of H_3PO_4, CH_3COOH, H_3BO_3 and 0.2 mol dm^{-3} NaOH) were of reagent grade (p. a.). Potassium chloride and KOH (reagent grade p. a.)

Table 1 Values of *m/z* of obtained oxidation products of compounds **1–4** from HPLC–ESI-MS/MS

	Taxifolin	Quercetin	Luteolin	Fisetin
3,4-DHBA	153, 109	153, 109	153, 109	153, 109
		[370, 355, 193 (3-TMSderivative)]		
3,4-Dihydroxybenzaldehyde	137			
2,4-DHBA				153, 137, 109
3,4-Dihydroxyphenyl oxoacetic acid		181, 153, 109		181, 153, 109
		[383, 281 (3-TMS derivative)]		
2,4-Dihydroxyphenyl oxoacetic acid				181, 165, 153, 137, 109
4-Hydroxyphenyl oxoacetic acid				165
4-Hydroxybenzaldehyde				121
2,4,6-THBA	169, 151, 125	169, 151, 125		
		[443, 353 (4-TMS derivative)]		
2,5,7-Trihydroxy-4*H*-1-benzopyran-4-one			193 [25]	
2,4,6-Trihydroxy-benzaldehyde			153, 125, 56	
2,4,6-Trihydroxyphenyl oxoacetic acid	197, 169, 151, 125	197, 169, 151, 125		
		[471, 443, 369, 73 (4-TMS derivative)]		
Open structure—acid	305, 167	305	305, 167	
		[666, 651, 355 (5-TMS)]		
Benzofuranone derivative		317, 289, 207, 151		301, 273, 191, 153, 135
		[678, 369, 281 (5-TMS derivative)]		
Open structure—aldehyde			289	273, 243, 163, 135, 109
2′-Hydroxytaxifolin	319, 181, 153, 125	N/A	N/A	N/A
Myricetin	317, 301	N/A	N/A	N/A
6-Hydroxyluteolin	N/A	N/A	301, 167, 125	N/A
Open structure—oxoacetic acid		333, 305, 261, 151		
Quinone	301			
Quinoid structure	N/A			283, 209, 187, 171, 136, 125
Quinone open structure—oxobenzaldehyde			315	
Open structure—oxobenzaldehyde			317, 269, 167, 166	
3-(3,4-Dihydroxyphenyl)-2-hydroxy-1-(2,4,6-trihydroxyphenyl)prop-2-en-1-one		303		
		[721, 647, 575 (6-TMS derivative)]		
Dimer		601, 302	569, 417, 285, 151	

Values obtained (for derivatized products) of **2** by GC–MS are in brackets

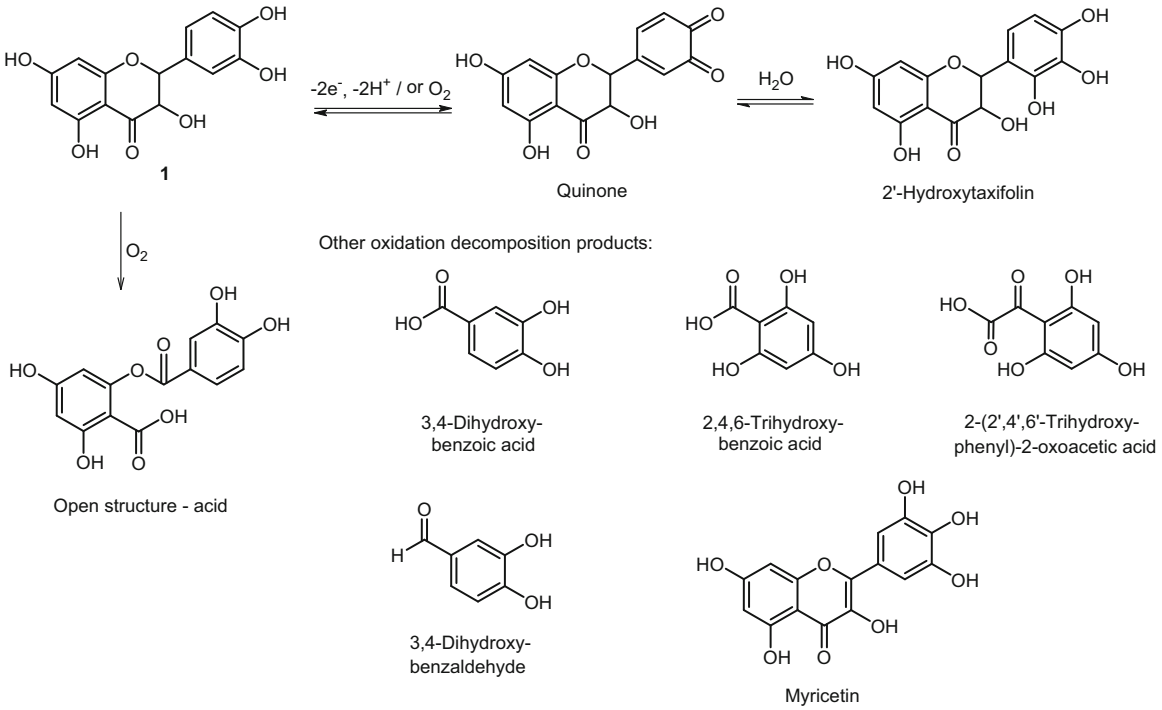
N/A not applicable

were used as 0.1 M and 0.36 mM solutions, respectively. The solutions were prepared with ultrapure water (Millipore). Methanol and acetonitrile were HPLC grade (Carlo Erba, Milan, Italy). 3,4,5-Trihydroxybenzoic acid (more than 99 % purity) was from Alpha Aesar (Lancaster, England); 3,4-dihydroxybenzoic acid (>97 % purity) and 2,4-dihydroxybenzoic acid (97 % purity) were from Sigma Aldrich (Milan, Italy). Standard solutions of all flavonoids and low molecular hydroxybenzoic acids for HPLC were prepared in methanol. All reagents and chemicals were used without any further purification.

Stability of compounds in solution: UV–Vis measurements

The 1.0-cm quartz cuvettes were used for recording the absorption spectra during testing the stability of compounds when exposed to the air oxygen. Spectral changes were registered using Agilent 8453 diode array UV–Vis spectrometer. The procedure of measurement of taxifolin stability in solution was as follows: the first absorption spectrum of solution prepared under inert atmosphere was recorded in a closed cuvette; then, absorption spectra were

Scheme 2



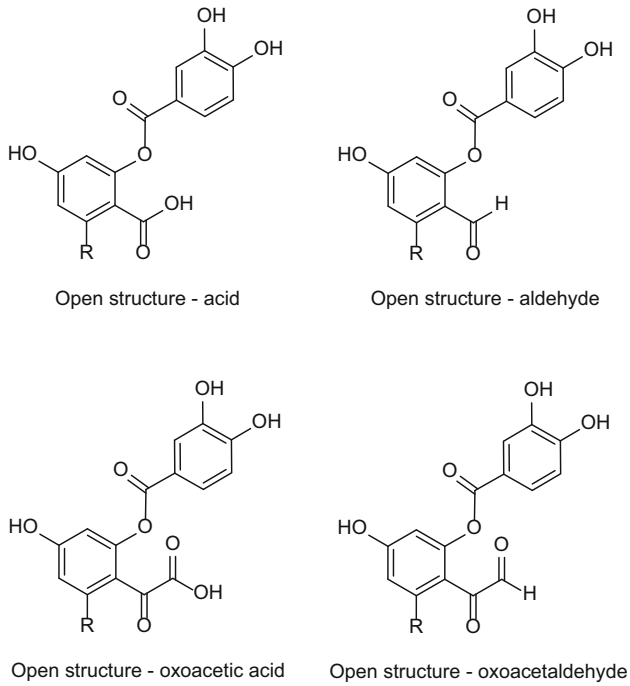


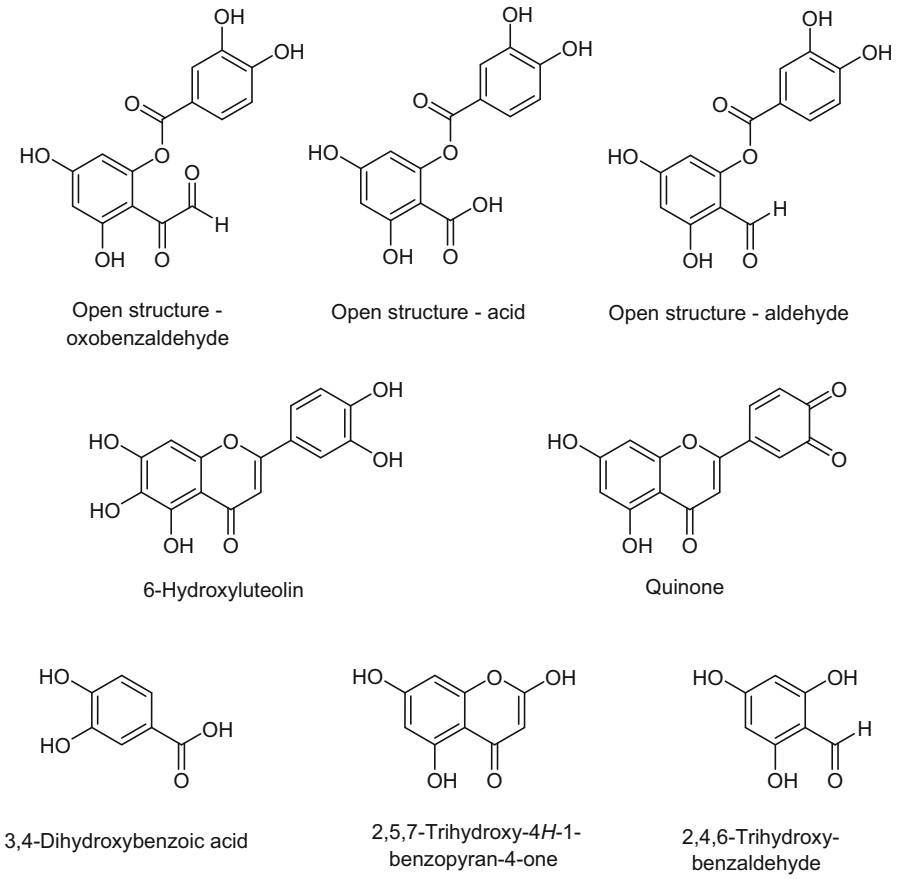
Fig. 4 Chemical structures of 'open structures' resulting from the flavonols and flavones oxidation by oxygen

measured after opening the cuvette and manually shaking of solution in the presence of air. Oxygen was removed from the solution by passing a stream of argon.

High-pressure liquid chromatography with photodiode array detector (HPLC–DAD)

A high-pressure liquid chromatography Agilent 1200 Series HPLC Systems equipped with diode array detector (Agilent Technologies) was used. The chromatographic separation was performed on analytical reverse phase C-8 column (HyPurity C8, 150 × 3 mm, 5 μm, Thermo Scientific, Dubuque, USA) connected to C-18 pre-column (HyPurity C18, 10 × 3 mm, 5 μm, Thermo Scientific, Dubuque, USA). Column temperature: 20 °C. The gradient elution programme used eluents (A): aqueous solution of 0.1 % H_3PO_4 and (B): acetonitrile. The gradient used for solutions of fisetin and luteolin was: 0–2 min, 95 % A; 2–30 min, linear gradient to 40 % A; 30–35 min, linear gradient to 30 % A; 35–60 min, linear gradient to 5 % A and 95 % B (setup 1). The gradient used for solutions of taxifolin and quercetin was: 0–2 min, 95 % A; 2–40 min, linear gradient to 40 % A; 40–45 min, linear gradient to 30 % A; 45–70 min, linear gradient to 5 % A and 95 % B

Scheme 3



(setup 2). The flow rate was 0.2 cm^3 min^{-1}, the injection volume was 40 mm^3. Diode array detector acquisition parameters were: acquisition range 190–800 nm, 2 nm step.

High-pressure liquid chromatography with electrospray ionization tandem mass spectrometer (HPLC–ESI-MS/MS)

HPLC–ESI-MS/MS was carried out using a 1200 Infinity HPLC (Agilent Technologies, USA), coupled to a Jet Stream ESI interface (Agilent Technologies) with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-TOF (Agilent Technologies). The chromatographic separation took place at 30 °C and was performed on an analytical reverse phase C-18 column (C18-extended 1.8 μm, 50 × 2.1 mm, Agilent Technologies, USA) connected to a C-18 pre-column (TC-C18 (2) 5 μm, 12.5 × 2.1 mm, Agilent Technologies, USA).

The eluents were: (A): 90 % H_2O with 0.1 % formic acid, and (B): 10 % acetonitrile with 0.1 % formic acid, and the flow rate was 0.2 cm^3 min^{-1}. Injection volume was 10 mm^3. The programme was: 90 % A and 10 % B for 2 min; then to 50 % A and 50 % B in 9 min; then linear gradient to 30 % A to 70 % B in 3 min; then to 10 % A to 90 % B in 4 min, hold for 4 min. Conditioning takes 3 min.

The ESI operating conditions were: drying gas (N_2, purity >98 %) 350 °C and 10 dm^3 min^{-1}; capillary voltage 4.5 kV; nebulizer gas 35 psig; sheath gas (N_2, purity >98 %) 375 °C and 11 dm^3 min^{-1}. The collision gas was nitrogen (purity 99.999 %). The fragmentor potential was 175 V and the collision energy for the MS/MS experiments ranged between 20 and 50 V depending on the target compound.

High-resolution MS and MS/MS spectra were achieved in negative mode in the range 100–3200 *m/z*; the mass axis was calibrated using the Agilent tuning mix HP0321 (Agilent Technologies) prepared in acetonitrile.

Gas chromatography

The products of quercetin oxidation were identified using a Trace GC gas chromatograph (Thermo Electron

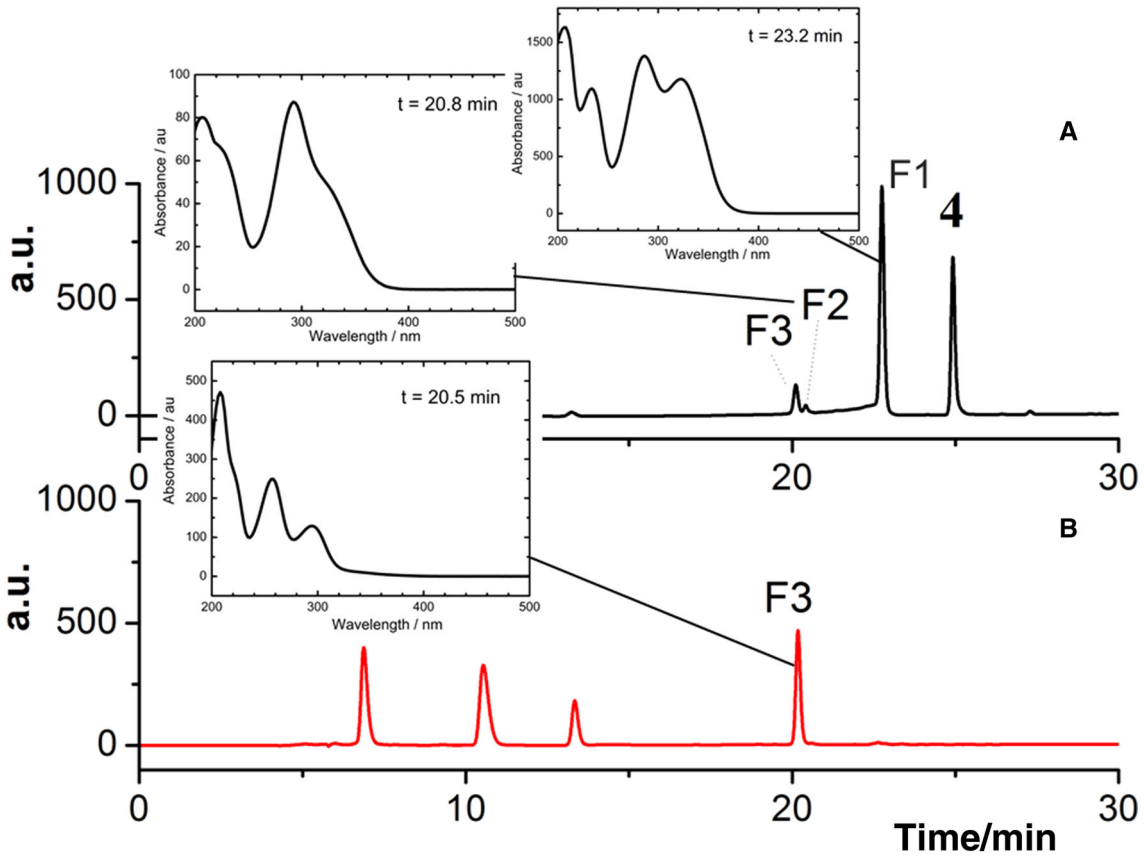
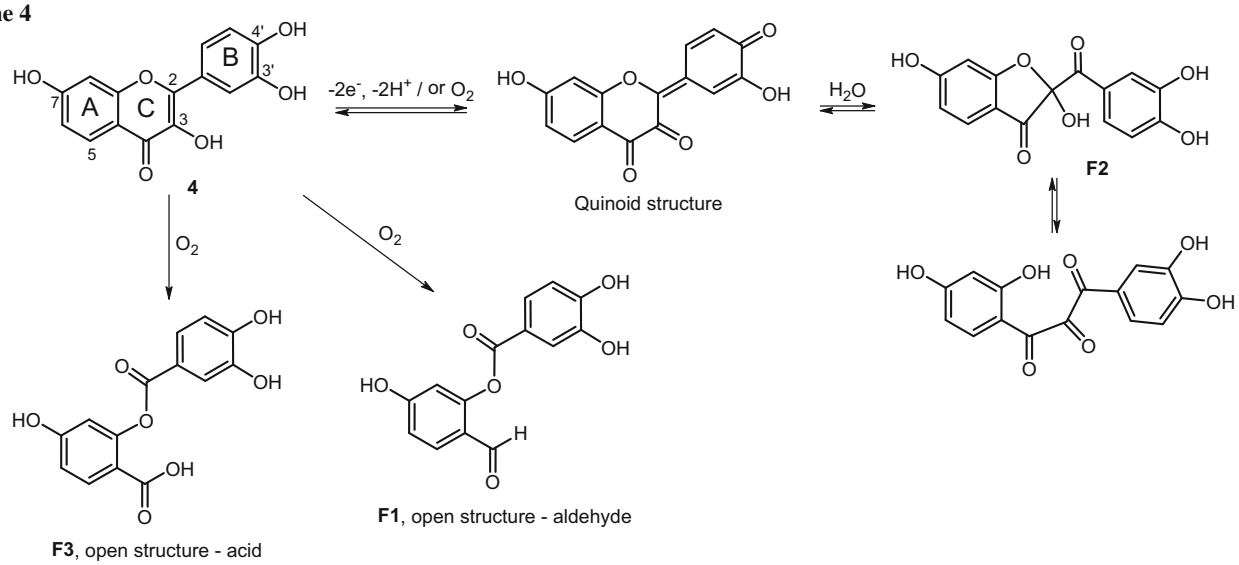


Fig. 5 HPLC chromatogram of fisetin in 0.1 M KCl and 3.6×10^{-3} M KOH: **a** prepared freshly under ambient conditions, **b** recorded in 60 min. Detector 280 nm. As *insets* are shown absorption spectra of oxidation products *F1–F3* after subtraction of column baseline

Scheme 4



Corporation, USA) equipped with a PTV injection port and a mass spectrometric detector based on an ion trap analyzer (Polaris Q, Thermo Electron Corporation, USA). The PTV injector was in the CT 'splitless with surge' mode at 280 °C with a surge pressure of 100 kPa, and the mass spectrometer parameters were: ion source temperature

230 °C, electronic impact ionization (70 eV), scan range *m/z* 50–700 and interface temperature 280 °C. Chromatographic separation was performed on a DB-5MS chemically bonded fused silica capillary column (J & W Scientific, Agilent Technologies) with stationary phase 5 % phenyl–95 % methylpolysiloxane, and of dimensions 0.25 mm ID, 0.1 μm film thickness, 25 and 30 m length. The gas chromatographic conditions were as follows: initial temperature 57 °C, 2 min isothermal, then ramped at 10 °C min^{-1} up to 200 °C, 3 min isothermal, then ramped at 20 °C min^{-1} up to 300 °C and then isothermal for 20 min. The carrier gas was He (purity 99.9995 %), at a constant flow rate of 1.2 cm^3 min^{-1}. The peak assignment was based on comparison with analytical reference compounds and materials, with library mass spectra (NIST 1.7) and with mass spectra reported in the literature.

To perform GC–MS analysis, oxidation products were derivatised with a silylating agent *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Derivatisation conditions were: 10 mm^3 of 2,4-dihydroxybenophenone (solution in isopropanol; internal standard IS1) was added to the sample; the solution was dried and 30 mm^3 of the derivatisation agent BSTFA in 50 mm^3 of AcOEt was added; the reaction took place 30 min at 60 °C in closed glass vials. Just before injection, 10 mm^3 of hexadecane (solution in isooctane; internal standard IS2) and 150 mm^3 of AcOEt were added; 2 mm^3 of the final solution was injected in the GC system.

Theoretical calculations

Quantum chemical calculations of organic compounds to obtain their UV–Vis spectrum were performed using the density functional theory (DFT) calculations with EDF2/6-31G* density functional model (Spartan'14, v.1.1.8). Their structures were optimized to the minimum of geometry. Calculations were done for molecules in vacuum.

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