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Ecofriendly synthesis of halogenated flavonoids and evaluation of their antifungal activity

Roberta Bernini,*[a] Marcella Pasqualetti,[b] Gianfranco Provenzano[a] and Sabrina Tempesta[b]

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Brominated and chlorinated flavonoids belonging to different classes (flavanones, flavones and catechins) were prepared from the corresponding flavonoids by a simple and ecofriendly procedure based on the use of sodium halides, aqueous hydrogen peroxide and acetic acid. Pure samples of substrates and products were tested against *Trichoderma koningii*, *Fusarium oxysporum* and *Cladosporium cladosporioides*, common saprotrophic soil and seed fungi, potential pathogens for humans and their activity was expressed as linear mycelial growth inhibition (%). Among them, 8-chloro-5,7,3′,4′-tetramethoxyepicatechin **29**, a novel catechin derivative, exhibited a remarkable effect against all tested fungi also at low concentrations.

Introduction

Flavonoids are more than 5000 plant secondary metabolites involved in important biological processes such as nitrogen fixation, photosensitization, energy transfer, plant growth, control of respiration, and photosynthesis.[1,2] In flowers they provide colors attractive to plant pollinators; in leaves they promote physiological survival of the plant, protecting it from pathogens and UV-B radiation. Flavonoids are found in fruits, vegetables, propolis, honey, tea, and wine and are of interest to human health because of their beneficial biological effects such as anti-inflammatory, antioxidant, antiviral, antibacterial, anticancer, and antimicrobial activities.[3–5]

Structurally, these compounds possess a common phenylbenzopyrone nucleus (C6–C3–C6) consisting of two aromatic rings (A and B) linked by an oxygenated central pyranic ring (C) and are categorized according to the saturation level of the central ring and the presence of carbonylic or alcoholic functionality. Among them, flavanones, flavones, and flavan-3-ols (catechins) are widely diffused in food and beverages (Fig. 1).

The introduction of one or more halogen atoms into the A or B-ring of flavonoids produced the corresponding halogenated derivatives useful as synthetic intermediates.[6–9] In addition, halogen atoms confer to flavonoids additional biological properties.[10,11] For example, a series of mono- and di-halogenated flavonoids have shown a remarkable antifungal activity against some human pathogens *e.g Trichophyton longifusus, Candida albicans, Aspergillus flavus, Microsporium canis, Fusarium solanii* and *Candida glaberata* even if a structure–activity relationship has not been described.[12–14]

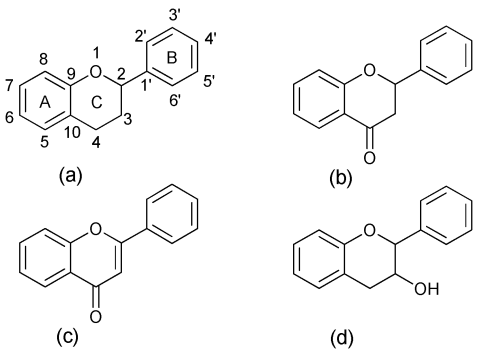


Fig. 1 Basic structure of (a) flavonoids; (b) flavanones; (c) flavones and (d) flavan-3-ols.

Generally, the halogenation of aromatic compounds is carried out using molecular halogens in chlorinated solvents. Even if efficient, this procedure has several environmental drawbacks due to the toxic and hazardous nature of reagents (chlorine gas and liquid bromine), and solvents. In addition, pollutant wastes were produced as by-products.

In order to overcome these problems, in the last few years several papers have reported green procedures based on the generation of the electrophilic reagent X^+ (*e.g.* Br^+, Cl^+) by an ecofriendly oxidation of halide ions. The most used benign oxidants are dimethyldioxirane (DMD), potassium peroxymonosulfate (Oxone®) and hydrogen peroxide (H_2O_2). Although DMD

[a] *Dipartimento di Scienze e Tecnologie per l'Agricoltura, le Foreste, la Natura e l'Energia (DAFNE), Università degli Studi della Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy. E-mail: berninir@unitus.it; Fax: +39 0761 357242; Tel: +39 0761 357452*

[b] *Dipartimento di Scienze Ecologiche e Biologiche (DEB), Università degli Studi della Tuscia, Largo dell'Università, 01100 Viterbo, Italy*

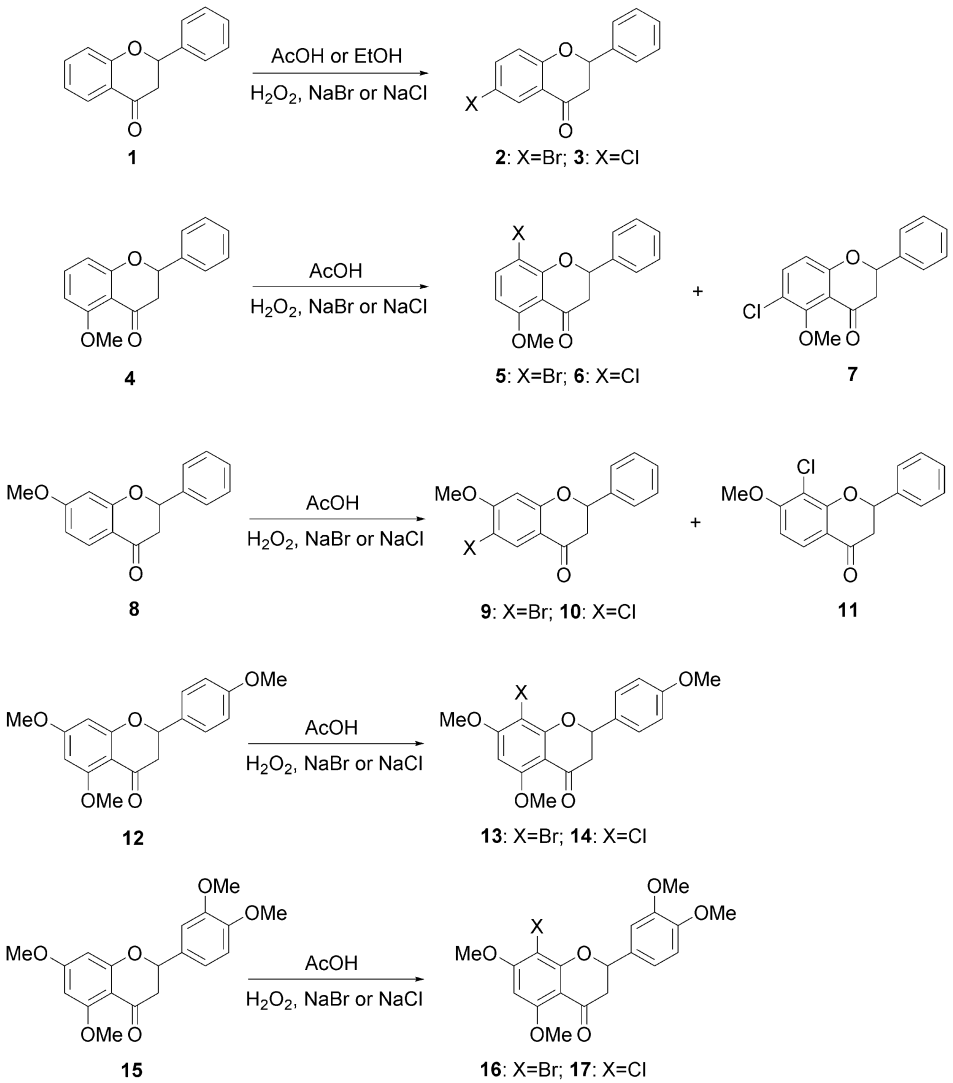
is an efficient oxidizing agent and its preparation is an easy and safe operation, Oxone® is more readily accessible being commercially available and H_2O_2 appears as the best candidate producing water as the only by-product.[15,16] In combination with hydrochloric acid or sodium halides, DMD and Oxone® were very efficient for the halogenation of phenols, methoxybenzenes[17–19] and flavonoids;[20,21] similarly, the selective halogenation of phenols and methoxybenzene derivatives was carried out providing the products in good yields using H_2O_2 in the presence of catalysts or in acetic acid.[22,23] To the best of our knowledge, no papers on the halogenation of flavonoids under similar conditions have been published.

As part of our research on the chemical modifications of natural phenols to obtain bioactive compounds by ecofriendly procedures,[24,25] we prepared several halogenated flavonoids using a simple and environmentally benign way based on the use of sodium halides, aqueous solution of hydrogen peroxide and acetic acid as a solvent. Final compounds were tested against common saprotrophic soil and seed fungi, potential pathogens for humans, *e.g. Trichoderma koningii*, *Fusarium oxysporum*, and *Cladosporium cladosporioides*.[26–28]

Results and discussion

Synthesis of halogenated flavonoids

We firstly investigated the efficiency of the selected halogenating system on flavanone **1**, 5-methoxyflavanone **4**, 7-methoxyflavanone **8**, 5,7,4′-trimethoxyflavanone (methylated naringenin) **12** and 5,7,3′,4′-tetramethoxyflavanone (methylated hesperetin) **15** (Scheme 1). All experimental data are reported in Table 1. In acetic acid, in the presence of NaX (X = Br, Cl) and H_2O_2 (30% water solution), flavanone **1** was converted into the corresponding 6-halogenated derivatives **2** and **3** in 70% and 62% yields (Table 1, entries 1 and 3). According to our previous results,[20] flavanone **1** was regioselectively brominated and chlorinated at position C-6 activated by the *para* ethereal oxygen of the heterocylic C-ring. In our hands, the bromination and chlorination of flavanone **1** failed in ethanol (Table 1, entries 2 and 4).



Scheme 1 Halogenation of flavanones **1**, **4**, **8**, **12** and **15**.

These results confirmed the crucial role of acetic acid in promoting the formation of the electrophilic species X^+ (Br^+ and Cl^+) responsible for the attack of the aromatic A-ring of flavanone and the success of halogenation.[22] In the presence of H_2O_2–NaX–CH_3COOH, the reaction proceeded with good regioselectivity also with activated substrates; the combination of the *ortho* and *para* effects of both the ethereal oxygen of the heterocylic C-ring and methoxyl groups present in the A-ring was responsible for the formation of the exclusive (or main) halogenated regioisomer. The bromination of 5-methoxyflavanone **4** produced the corresponding 8-bromoderivative **5** in quantitative yield (Table 1, entry 5); chlorination gave 8-chloroderivative **6** as the main product (72%) and 6-chloroderivative **7** as the secondary product (Table 1, entry 6). Similarly, 7-methoxyflavanone **8** afforded the corresponding 6-bromoderivative **9** in quantitative yield (Table 1, entry 7); 6-chloroderivative **10** and 8-chloderivatives **11**, respectively, in 70 and 18% yields (Table 1, entry 8). Finally, methylated naringenin **12** and methylated hesperetin **15** gave the corresponding 8-bromo and 8-chloroderivatives **13** and **14**, respectively, in 95 and 85% yields; **16** and **17** in 88 and 76% yields (Table 1, entries 9–12). Compared to DMD/HCl and Oxone®/NaX systems, halogenated flavanones were obtained in better yields with the only exception of 6-bromo and 6-chloroflavanones **2** and **3**. The absence of 6,8-dihalogenated derivatives evidencing a better control of the regioselectivity of reaction is worth noting.

On the basis of these experimental results, we extended the use of the H_2O_2–NaX–CH_3COOH system to biologically relevant flavonoids such as 5,7-dimethoxyflavone (chrysin) **18** and catechin derivatives **21** and **26**. As depicted in Scheme 2, compound **18** was converted into the corresponding 8-bromo and 8-chloroderivatives **19** and **20** in 92 and 74% yields (Table 1, entries 13 and 14) whereas 5,7,3′,4′-tetramethylated catechin **21** and 5,7,3′,4′-tetramethylepicatechin **26** resulted in being more reactive producing, respectively, the corresponding 8-halogenated compound **22**, **24**, **27**, **29** as main products and 6,8-derivatives **23**, **25**, **28**, **30** as secondary products (Table 1, entries 15–18).

These data were appealing because the H_2O_2–NaX–CH_3COOH system resulted in being efficient with different classes of flavonoids producing the corresponding halogenated derivatives in good to excellent yields. In addition, to the best of our knowledge, catechin derivatives **22–25** and **27–30** are novel compounds.

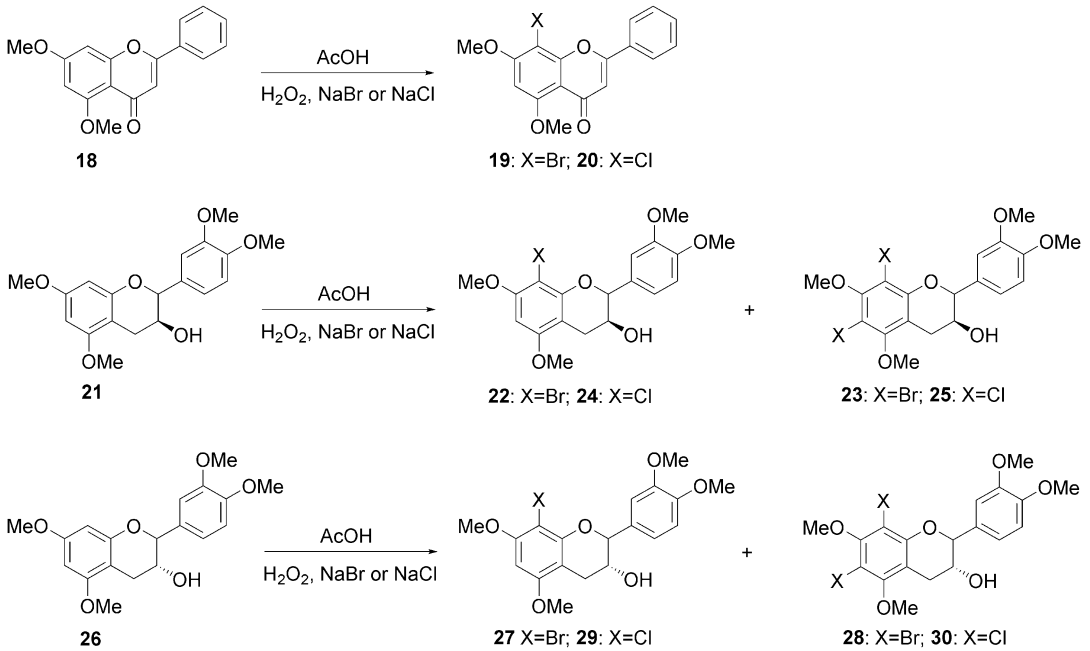
Evaluation of the antifungal activity

Pure samples of flavonoids **4**, **8**, **12**, **15**, **18**, **21**, **26** and the corresponding halogenated derivatives **5**, **6**, **9**, **10**, **11**, **13**, **14**, **16**, **17**, **19**, **20**, **22**, **23**, **24**, **25**, **27**, **28** and **29** were screened *in vitro* at three different concentrations 0.5, 2.0 and 8.0×10^{-4} M[13,14,25] for their growth inhibitory activity against *Trichoderma koningii*, *Fusarium oxysporum* and *Cladosporium cladosporioides*, common saprotrophic soil and seed fungi, potential pathogens for humans.[26–28] All experimental results, expressed as linear mycelial growth inhibition (%), are reported in Table 2 and grouped in the MDS plot depicted in Fig. 2. The MDS approach showed the main distribution trend of the flavonoids along the first axis related to their antifungal activity that increases from right to left. 7-Methoxyflavanone **8** and 8-chloro-5,7,3′,4′-tetramethoxyepicatechin **29**, located on the right side of the plot (group A), were the most active compounds against all species tested and the percentage of radial growth inhibition was high also at a lower tested concentration (0.5×10^{-4} M) against *Trichoderma koningii* (respectively, 49.4% and 51.5%, Table 2). 8-Bromo-5-methoxyflavanone **5**, 6-bromo-7-methoxyflavanone **9**, 8-chloro methylated naringenin **14**, methylated hesperetin **15**, 8-bromo methylated hesperetin **16**, 8-chloro methylated hesperetin **17**, 8-bromo methylated chrysin **19**, 8-bromo-5,7,3′,4′-tetramethoxycatechin **22**, 6,8-dibromo-5,7,3′,4′-tetramethoxycatechin **23**, 8-chloro-5,7,3′,4′-tetramethoxycatechin **24**, 6,8-dichloro-5,7,3′,4′-tetramethoxycatechin **25** and 6,8-dibromo-5,7,3′,4′-tetramethoxyepicatechin **28** (group D) showed low antifungal activity against all tested fungi (mean growth reduction <25%, Table 2). Flavonoids distributed in the central part of the plot showed a relatively high activity and could be

Table 1 Halogenation of flavonoids depicted in Schemes 1 and 2

Entry	Substrate[a]	Experimental conditions	Product[a] (yield%)
1	**1**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 24 h	**2**: 70
2	**1**	H_2O_2 (3.0 eq.), EtOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 24 h	**2**: —
3	**1**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), NaCl (1.0 eq.), 40 °C, 48 h	**3**: 62
4	**1**	H_2O_2 (6.0 eq.), EtOH (2.5 mL), NaCl (1.0 eq.), 40 °C, 48 h	**3**: —
5	**4**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 4 h	**5**: 98
6	**4**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaCl (3.0 eq.), 25 °C, 24 h	**6**: 72; **7**: 20
7	**8**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 4 h	**9**: 95
8	**8**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), NaCl (6.0 eq.), 40 °C, 48 h	**10**: 70; **11**: 18
9	**12**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 4 h	**13**: 95
10	**12**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), NaCl (6.0 eq.), 25 °C, 8 h	**14**: 85
11	**15**	H_2O_2 (1.0 eq.), AcOH (2.5 mL), NaBr (3.0 eq.), 25 °C, 4 h	**16**: 88
12	**15**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), NaCl (6.0 eq.), 25 °C, 24 h	**17**: 76
13	**18**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 24 h	**19**: 92
14	**18**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), NaCl (6.0 eq.), 40 °C, 24 h	**20**: 74
15	**21**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 4 h	**22**: 70; **23**: 22
16	**21**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), NaCl (6.0 eq.), 40 °C, 4 h	**24**: 60 **25**: 18
17	**26**	H_2O_2 (3.0 eq.), AcOH (2.5 mL), NaBr (1.0 eq.), 25 °C, 4 h	**27**: 78; **28**: 20
18	**26**	H_2O_2 (6.0 eq.), AcOH (2.5 mL), AcOH, NaCl (10.0 eq.), 25 °C, 4 h	**29**: 62; **30**: 10

[a] Calculated after chromatographic purification.



Scheme 2 Halogenation of flavonoids **18**, **21** and **26**.

Table 2 Antifungal activity of tested flavonoids expressed as inhibition (%) of linear mycelial growth; significant differences at $P < 0.05$; no significant values are unreported

	Trichoderma koningii			*Fusarium oxysporum*			*Cladosporium cladosporioides*		
Flavonoid	8.0×10^{-4} M	2.0×10^{-4} M	0.5×10^{-4} M	8.0×10^{-4} M	2.0×10^{-4} M	0.5×10^{-4} M	8.0×10^{-4} M	2.0×10^{-4} M	0.5×10^{-4} M
4	45.3	43.0	41.1	29.6	28.4	21.4	47.1	36.8	25.6
5	15.1	16.3	21.3	19.2	15.1	16.3	21.2	15.0	13.2
6	48.4	25.0	23.2	22.3	20.4	12.5	26.5	23.1	17.4
8	82.2	70.7	49.4	55.8	49.7	27.1	70.3	49.6	26.4
9	21.9	13.1	5.0	11.1	6.9	6.7	7.8	14.8	10.5
10	48.9	34.6	31.3	28.6	35.4	24.4	38.3	32.0	20.7
11	43.3	30.2	28.3	24.4	18.9	12.3	23.9	25.1	5.8
12	74.6	61.5	18.4	60.0	52.8	14.1	17.4	18.6	10.2
13	70.4	20.8	10.3	27.5	18.7	14.9	24.1	9.0	—
14	22.5	15.6	—	20.4	20.7	15.5	7.8	—	—
15	8.9	10.7	6.9	15.2	12.5	9.8	6.7	7.3	—
16	19.5	—	4.9	12.3	21.7	10.8	16.9	8.6	4.7
17	15.9	—	5.9	13.9	17.4	8.3	8.1	—	—
18	52.9	59.5	34.5	27.0	23.7	18.2	28.2	21.5	16.5
19	23.6	21.1	—	17.1	10.2	—	12.6	—	—
20	80.2	48.1	32.4	41.8	26.5	14.9	47.7	18.9	13.8
21	38.2	48.4	22.8	16.4	18.2	9.6	14.1	17.1	11.3
22	23.7	5.3	21.0	22.8	7.1	6.8	16.5	4.5	—
23	20.0	17.4	6.7	40.6	26.0	5.7	10.8	8.6	5.1
24	32.8	17.5	6.5	—	4.2	—	13.3	5.6	—
25	29.3	23.8	6.8	9.3	4.5	6.8	—	—	—
26	70.2	12.5	—	36.3	10.2	—	48.9	21.5	5.9
27	68.7	66.9	22.4	32.5	22.3	6.5	40.2	27.3	17.6
28	25.4	29.0	22.8	6.2	5.9	—	—	—	—
29	73.2	72.2	51.5	43.7	39.3	19.7	55.2	47.4	19.3

separated in two main groups: group B including 8-chloro-5-methoxyflavanone **6**, 8-chloro-7-methoxyflavanone **11** and 5,7,3′,4′-tetramethoxycatechin **21**, active mainly against *Trichoderma koningii*; group C including 5-methoxyflavanone **4**, 6-chloro-7-methoxyflavanone **10**, methylated chrysin **18**, 8-chloro-5,7-methylated chrysin **20** and 8-bromo-5,7,3′,4′-tetramethoxyepicatechin **27**, active against all fungal species. Finally, methylated naringenin **12** resulted in being active only against *Trichoderma koningii* and *Fusarium oxysporum*; 8-bromo methylated naringenin **13** and 5,7,3′,4′-tetramethoxyepicatechin **26** were active, respectively, against *Trichoderma koningii* and all fungi but only at higher concentrations.

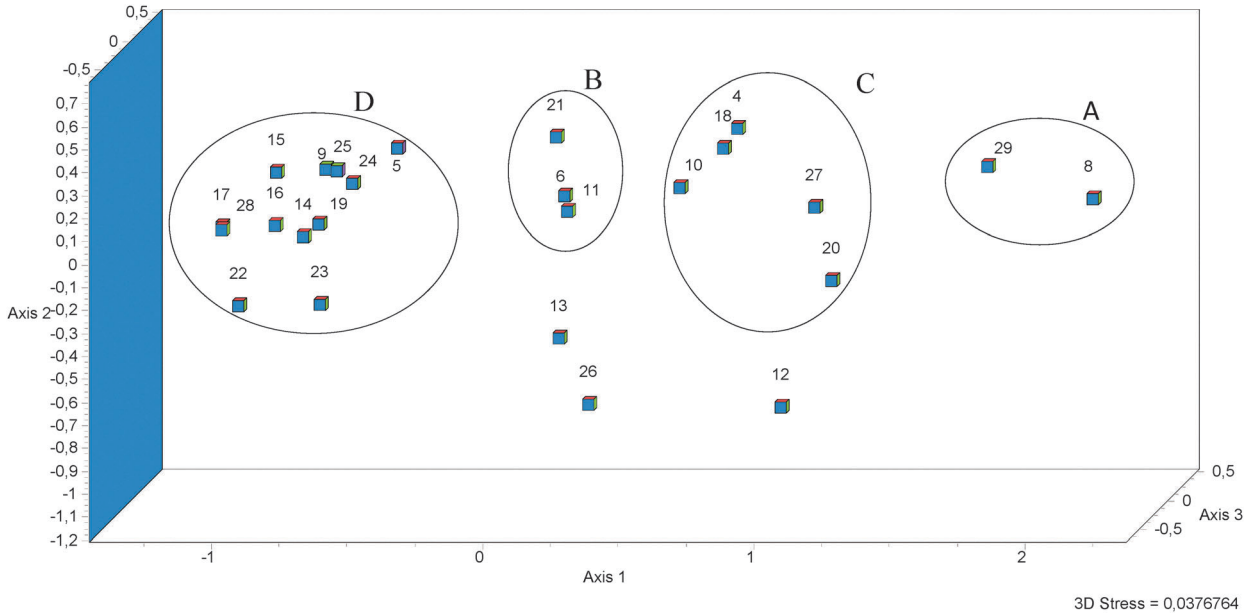


Fig. 2 MDS analysis on fungal activity of tested compounds.

Conclusions

A simple and environmentally benign bromination and chlorination procedure based on the use of aqueous solutions of hydrogen peroxide, sodium halides (NaBr and NaCl) and acetic acid was applied for the first time to flavonoids to obtain the corresponding halogenated derivatives, useful synthetic intermediates and potentially bioactive compounds. The procedure was efficient with different representative classes of flavonoids; the halogenation proceeded with good regioselectivity induced by a combination of the effects of both the ethereal oxygen of the heterocylic C-ring and methoxyl groups present in the aromatic A-ring. To the best of our knowledge, catechin derivatives **22–25** and **27–30** appear as novel compounds. Substrates and the corresponding halogenated derivatives were tested against *Trichoderma koningii*, *Fusarium oxysporum*, and *Cladosporium cladosporioides*, common saprotrophic soil and seed fungi, potential pathogens for humans. Although experimental data did not evidence a structure–activity relationship, almost all flavonoids showed an inhibitory activity; in addition the effect of 8-chloro-5,7,3′,4′-tetramethoxyepicatechin **29** which resulted in being active against all tested fungi also at low concentrations is remarkable.

Experimental section

Materials and methods

All reagents and flavonoids were of the highest grade available and used as such (Sigma-Aldrich, Milan, Italy). Chromatographic purifications were performed on columns packed with a Merck silica gel 60, 230–400 mesh. Thin layer chromatography was carried out using Merck platen Kieselgel 60 F254. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 200 and 400 MHz. Mass spectra were recorded using a gas chromatograph Shimadzu GC-17A equipped with an electron beam of 70 eV, a SPB column (25 m × 0.30 mm and 0.25 mm film thickness) and a mass-selective detector QP 6000. The injector temperature was 280 °C. An isothermal temperature profile of 60 °C for 5 min, followed by a 10 °C min^{-1} temperature gradient to 250 °C for 10 min, was used. Chromatographic grade helium was used as the carrier gas. High Resolution Mass Spectrometry (HRMS) analyses were performed using a Micromass Q-TOF micro Mass Spectrometer (Waters).

Methylation reactions

5,7,4′-Trimethoxyflavanone (methylated naringenin) **12**; 5,7,3′,4′-tetramethoxyflavanone (methylated hesperetin) **15**; 5,7,3′,4′-tetramethylcatechin **21** and 5,7,3′,4′-tetramethylepicatechin **26** were prepared as already reported by us.[29,30]

5,7,4′-Trimethoxyflavanone (methylated naringenin) (12)

Yield: 90%; colorless oil; spectroscopic data are according to those reported in the literature.[29]

5,7,3′,4′-Tetramethoxyflavanone (methylated hesperetin) (15)

Yield: 88%; colorless oil; spectroscopic data are according to those reported in the literature.[29]

5,7,3′,4′-Tetramethoxycatechin (21)

Yield: 92%; yellow oil; spectroscopic data are according to those reported in the literature.[30]

5,7,3′,4′-Tetramethoxyepicatechin (26)

Yield: 90%; yellow oil; spectroscopic data are according to those reported in the literature.[30]

Halogenation reactions

A 30% aqueous solution of hydrogen peroxide (3.0–6.0 eq.) was added to the solution of flavonoid (0.5 mmol) and sodium halide (1.0–3.0 eq.) in acetic acid (2.5 mL); then, the mixture was stirred at room temperature or 40 °C for 4–48 h depending on the substrate. The reaction was monitored by thin layer chromatography (TLC) and gas-mass chromatography (GC-MS). At the end, the crude was treated with sodium thiosulfate and extracted with ethyl acetate (3 × 10 mL). The reunited organic fractions were dried over anhydrous sodium sulfate; after filtration, the solvent was evaporated under reduced pressure. Final products were isolated and purified by chromatographic column using CH_2Cl_2/CH_3OH = 9.5/05 as an eluent and characterized by ^{1}H NMR, ^{13}C NMR, and HR-MS analysis.

6-Bromoflavanone (2)

Yield: 70%; yellow oil; spectroscopic data are according to those reported in the literature.[20]

6-Chloroflavanone (3)

Yield: 62%; colorless oil; spectroscopic data are according to those reported in the literature.[20]

8-Bromo-5-methoxyflavanone (5)

Yield: 98%; Yellow oil; spectroscopic data are according to those reported in the literature.[20]

8-Chloro-5-methoxyflavanone (6)

Yield: 72%; yellow oil; spectroscopic data are according to those reported in the literature.[20]

6-Chloro-5-methoxyflavanone (7)

Yield: 20%; colorless oil; spectroscopic data are according to those reported in the literature.[20]

6-Bromo-7-methoxyflavanone (9)

Yield: 95%; colorless oil; spectroscopic data are according to those reported in the literature.[20]

6-Chloro-7-methoxyflavanone (10)

Yield: 70%; yellow oil; spectroscopic data are according to those reported in the literature.[20]

8-Chloro-7-methoxyflavanone (11)

Yield: 18%; colorless oil; spectroscopic data are according to those reported in the literature.[20]

8-Bromo-5,7,4′-trimethoxyflavanone (13)

Yield: 95%; colorless oil; spectroscopic data are according to those reported in the literature.[20]

8-Chloro-5,7,4′-trimethoxyflavanone (14)

Yield: 85%; colorless oil; spectroscopic data are according to those reported in the literature.[20]

8-Bromo-5,7,3′,4′-tetramethoxyflavanone (16)

Yield: 88%; yellow oil; spectroscopic data are according to those reported in the literature.[31]

8-Chloro-5,7,3′,4′-tetramethoxyflavanone (17)

Yield: 76%; colorless oil; spectroscopic data are according to those reported in the literature.[31]

8-Bromo-5,7-dimethoxyflavone (19)

Yield: 92%; colorless oil; spectroscopic data are according to those reported in the literature.[32]

8-Chloro-5,7-dimethoxyflavone (20)

Yield: 74%; colorless oil; spectroscopic data are according to those reported in the literature.[32]

8-Bromo-5,7,3′,4′-tetramethoxycatechin (22)

Yield: 70%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.41–2.54 (dd, J_1 = 8.1 Hz, J_2 = 18.3 Hz, CH), 2.82–2.94 (dd, J_1 = 5.3 Hz, J_2 = 18.4 Hz, 1H, CH), 3.70 (s, 3H, OCH_3), 3.86 (s, 9H, $3OCH_3$), 4.01–4.11 (m, 1H, CH), 4.75 (d, J = 7.7 Hz, 1H, CH), 6.13 (s, 1H, Ph-H), 6.83–6.96 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 161.8, 148.5, 148.1, 134.5, 134.0, 120.5, 113.9, 110.9, 110.6, 93.6, 80.2, 79.5, 73.1, 57.0, 56.1, 55.8, 46.9, 35.2. $C_{19}H_{21}BrO_6$ requires C, 53.66; H, 4.98; O, 22.57. Found: C, 53.02; H, 5.01; O, 22.67.

6,8-Dibromo-5,7,3′,4′-tetramethoxycatechin (23)

Yield: 22%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.80–2.92 (dd, J_1 = 8.5 Hz, J_2 = 16.6 Hz, CH), 3.07–3.19 (dd, J_1 = 5.2 Hz, J_2 = 16.6 Hz, 1H, CH), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.86 (s, 6H, $2OCH_3$), 4.10–4.18 (m, 1H, CH), 4.75 (d, J = 7.7 Hz, 1H, CH), 6.83–6.86 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 162.7, 157.6, 149.7, 149.5, 134.1, 120.1, 112.6, 111.3, 107.6, 100.3, 99.4, 83.1, 67.1, 60.5, 59.9, 56.1, 56.0, 55.9, 27.5. $C_{19}H_{20}Br_2O_6$ requires C, 45.26; H, 4.00; O, 19.04. Found: C, 45.89; H, 4.09; O, 18.87.

8-Chloro-5,7,3′,4′-tetramethoxycatechin (24)

Yield: 60%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.42–2.56 (dd, J_1 = 8.1 Hz, J_2 = 18.3 Hz, CH), 2.80–2.94 (dd, J_1 = 5.3 Hz, J_2 = 18.4 Hz, CH), 3.72 (s, 3H, OCH_3), 3.86 (s, 9H, $3OCH_3$), 4.00–4.12 (m, 1H, CH), 4.72 (d, J = 7.7 Hz, 1H, CH), 5.83 (s, 1H, CH), 6.85–6.98 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 161.8, 148.5, 148.1, 134.5, 134.0, 120.5, 113.9, 110.9, 110.6, 93.6, 80.2, 79.5, 73.1, 57.0, 56.1, 55.8, 46.9, 35.2. $C_{19}H_{21}ClO_6$ requires C, 59.92; H, 5.56; O, 25.21. Found: C, 60.02; H, 5.26; O, 24.99.

6,8-Dichloro-5,7,3′,4′-tetramethoxycatechin (25)

Yield: 18%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.78–2.90 (dd, J_1 = 8.5 Hz, J_2 = 16.6 Hz, 1H, CH), 3.06–3.20 (dd, J_1 = 5.2 Hz, J_2 = 16.6 Hz, 1H, CH), 3.74 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.86 (s, 6H, $2OCH_3$), 4.10–4.20 (m, 1H, CH), 4.76 (d, J = 7.7 Hz, 1H, CH), 6.82–6.88 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 150.7, 148.8, 148.2, 134.9, 134.0, 120.6, 113.9, 110.8, 105.5, 97.8, 79.8, 79.6, 73.2, 59.5, 60.0, 55.9, 55.8, 55.7, 37.0. $C_{19}H_{20}Cl_2O_6$ requires C, 54.95; H, 4.85; O, 23.12. Found: C, 55.25; H, 4.98; O, 23.42.

8-Bromo-5,7,3′,4′-tetramethoxyepicatechin (27)

Yield: 78%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.85–2.88 (m, 2H, CH_2), 3.71 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.85 (s, 9H, 3OCH_3), 3.88 (s, 9H, 3OCH_3), 4.21–4.24 (m, 1H, CH), 4.91 (d, J = 7.7 Hz, 1H, CH), 6.13 (s, 1H, Ph-H), 6.85–7.05 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 161.9, 148.5, 148.2, 134.7, 134.2, 120.8, 113.9, 110.9, 110.8, 93.6, 80.4, 79.6, 73.1, 57.2, 56.0, 55.9, 46.8, 35.4. $C_{19}H_{21}BrO_6$ requires C, 53.66; H, 4.98; O, 22.57. Found: C, 54.02; H, 5.04; O, 22.87.

6,8-Dibromo-5,7,3′,4′-tetramethoxyepicatechin (28)

Yield: 20%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.85–2.88 (m, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.86 (s, 6H, 2OCH_3), 4.10–4.18 (m, 1H, CH), 4.75 (d, J = 7.7 Hz, 1H, CH), 6.83–6.86 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 162.8, 157.6, 149.7, 149.5, 134.1, 120.1, 112.6, 111.3, 107.6, 100.4, 99.4, 83.2, 67.2, 60.4, 59.9, 56.2, 56.1, 55.9, 27.4. $C_{19}H_{20}Br_2O_6$ requires C, 45.26; H, 4.00; O, 19.04. Found: C, 45.79; H, 4.12; O, 18.87.

8-Chloro-5,7,3′,4′-tetramethoxyepicatechin (29)

Yield: 62%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.85–2.88 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 3.86 (s, 9H, 3OCH_3), 4.02–4.12 (m, 1H, CH), 4.74 (d, J = 7.7 Hz, 1H, CH), 5.88 (s, 1H, Ph-H), 6.86–7.00 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 157.8, 148.5, 148.1, 137.9, 134.2, 120.6, 113.9, 110.8, 102.5, 92.8, 80.4, 79.8, 73.0, 57.0, 56.2, 55.9, 55.8, 54.2, 34.8. $C_{19}H_{21}ClO_6$ requires C, 59.92; H, 5.56; O, 25.21. Found: C, 60.02; H, 5.26; O, 24.99.

6,8-Dichloro-5,7,3′,4′-tetramethoxyepicatechin (30)

Yield: 10%; yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.85–2.88 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.86 (s, 6H, 2OCH_3), 4.12–4.18 (m, 1H, CH), 4.76 (d, J = 7.7 Hz, 1H, CH), 6.80–6.86 (m, 3H, Ph-H); ^{13}C NMR ($CDCl_3$), δ: 150.6, 148.6, 148.0, 134.8, 134.5, 120.7, 113.9, 110.8, 105.5, 97.8, 79.9, 79.8, 73.0, 59.9, 59.7, 56.2, 56.0, 55.9, 37.3. $C_{19}H_{20}Cl_2O_6$ requires C, 54.95; H, 4.85; O, 23.12. Found: C, 55.01; H, 4.75; O, 23.25.

Assay of antifungal activity

Flavone and flavanone exhibit fungicidal activity at 10^{-3}–10^{-5} M.[33,34] Therefore, in this study flavonoid derivatives were tested at 0.5, 2.0 and 8.0 $\times$ 10^{-4} M.[13,14,25] Each flavonoid was dissolved in acetone so that the final concentration of solvent in the test medium did not exceed 1% of the total solution composition; three solutions were prepared (0.5, 2.0 and 8.0 $\times$ 10^{-4} M). *T. koningii*, *F. oxysporum* and *C. cladosporioides* were used for the antifungal tests. Before testing, each isolate was subcultured on MEA (DIFCO) to ensure optimal growth characteristics and purity. The isolates were grown for 4–14 days on MEA at 25 °C. Conidia suspensions were prepared in sterile water supplemented with 0.01% of Tween 80. Each suspension was diluted to obtain the final inoculum, which ranged from 0.5 $\times$ 10^4 to 1.0 $\times$ 10^4 CFU mL^{-1}. The inoculum size was determined microscopically using Bürker's chamber and verified by plating 100 μL of serial dilutions of each inoculum onto an MEA plate and incubation until growth became visible. Each Petri dish (90 mm) containing 12 mL of the medium including the products in required concentrations (added to the agar at temperature below 50 °C) was inoculated with 2 μL of the inoculum suspensions; 5–6 replications for each concentration and fungus were made. The Petri dishes were incubated at 25 °C in the dark to a clearly visible growth. Evaluation of linear growth was conducted by measuring mycelial diameters of each inoculated plate at broadest, medium and smallest diameters, and compared with the corresponding control. The inhibition (%) of linear mycelial mean growth of *T. koningii*, *C. cladosporioides* and *F. oxysporum* was calculated after incubation for 3, 5 and 6 days respectively. The data were evaluated by analysis of variance, probability of single differences was calculated at the 5% level. The data were also ordered by Multi-Dimensional Scaling (MDS) analysis using SYSTAT 10.0 Statistics[35,36] to group the antifungal activity of all tested compounds; an input matrix of the Euclidean distance (calculated on linear mycelial growth) and the Kruskal loss function was utilized.[37]

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