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Activity of myricetin and other plant-derived polyhydroxyl compounds in human LDL and human vascular endothelial cells against oxidative stress

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ABSTRACT

Studies indicate that oxidative modifications of endothelium and LDL play a preeminent role in atherogenesis; therefore, the preservation of the endothelial antioxidant capacity and the inhibition of LDL oxidation by use of plant-derived compounds are an appealing strategy against several vascular disorders. On this basis, baicalein, eupatorin, galangin, magnolol, myricetin, oleuropein, silibinin and bilobalide were studied against various oxidative conditions. The radical scavenging capacity was analysed using DPPH and ORAC assays. Furthermore, the LDL oxidation was detected by measuring the formation of thiobarbituric acid reactive substances (TBARS) and by monitoring the oxidation kinetics. Further, we used cultured HUVEC to investigate the activities of the polyhydroxyl compounds towards the oxidative stress induced by H_2O_2. The lowest levels of TBARS were observed in the presence of oleuropein and baicalein, while myricetin, magnolol and eupatorin inhibited these ones to a lesser extent. In addition, oleuropein and myricetin exhibited higher protection in copper-induced LDL oxidation kinetics. However, only myricetin and galangin showed significant protective effects against H_2O_2 oxidative injury in HUVEC cells. Taken all together the results indicate myricetin as the most active agent among the selected plant-derived polyhydroxyl compounds, with prominent capacities against ox-LDL and ROS production in HUVEC.

1. Introduction

Inflammation, oxidative stress and endothelial dysfunction are associated with the pathogenesis of atherosclerosis [1]. Evidence supports the crucial role of oxidized low-density lipoproteins (ox-LDL) in the early inflammatory stage of atherosclerotic lesions [2,3]. Polyhydroxyl compounds are naturally occurring constituents of vegetable food and are main plant compounds with antioxidant activity. Epidemiological and clinical studies suggest that a polyphenol-rich diet may protect against cardiovascular disease and, generally, from pathological damage related to oxygen-derived free radicals (ROS) [4–6]; but the role of these plant-derived compounds in therapy is still matter of debate [7]. Generally, the epidemiological findings suggest the effectiveness of antioxidant-rich foods but, on the other hand, interventional trials with various vitamins as antioxidants, *i.e.* ß-carotene, vitamin E, ascorbic acid, folic acid, and related meta-analysis for the most part failed to demonstrate their usefulness [8,9]. In this context, it seems likely that other types of substances, as the polyphenols, may be the active compounds of vegetable foods useful against ROS-related diseases. Authors observed the necessity of proper experimental studies on single antioxidant compound, as prerequisite for clinical trials. On this basis, we selected eight polyhydroxyl compounds, mainly flavonoid derivatives, as baicalein, eupatorin, galangin, and myricetin, but also other compounds having different chemical structures, as bilobalide, magnolol, oleuropein, and silibinin (Table 1), to assess their antioxidant activities. Thus, the aim of this study was to find active compounds against oxidative stress for the potential use against atherosclerotic diseases. This study provided insight on the effects of the

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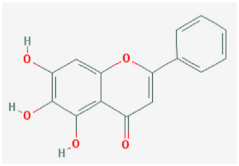
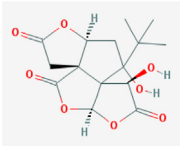
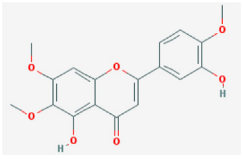
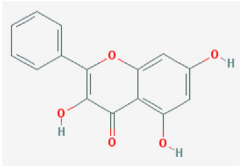
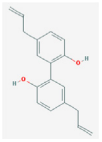
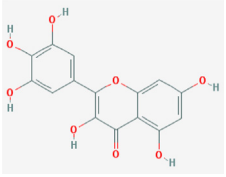
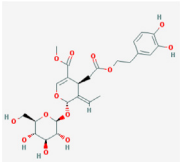
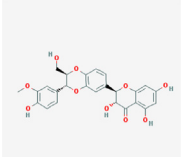
Table 1
Plant-derived compounds known for their presence in several botanicals.

Compound	Chemicals	Medicinal plant[a]	Plant parts	Chemical structure[b]
Baicalein	Flavone	*Scutellaria baicalensis*, *Oroxylum indicum*	Herb Leaf Stem bark Root	
Bilobalide	Sesquiterpene	*Ginkgo biloba*	Leaf	
Eupatorin	Flavone	*Eupatorium* spp. *Orthosiphon aristatus*	Herb Rhizome Root	
Galangin	Flavonol	*Alpinia* spp., *Helichrysum aureonitens*, propolis	Herb Rhizome	
Magnolol	Neolignan	*Magnolia officinalis*	Bark	
Myricetin	Flavonol	*Myrica rubra* *Tilia* spp.	Flower Leaf	
Oleuropein	Secoiridoid	*Olea europaea*	Fruit Leaf	
Silibinin	Flavanolignan	*Silybum marianum*	Fruit Herb	

[a] Examples of plants containing the compound.
[b] The chemical structures are from PubChem.

selected plant-derived compounds, showing myricetin as the most active antioxidant among the compounds considered, with prominent capacities to counteract LDL oxidation and ROS production.

2. Materials and methods

2.1. LDL isolation from human plasma

Blood was collected from healthy normolipidemic volunteers, which gave their informed consent before the venipuncture, after overnight fasting into vacutainer tubes containing Na_2-EDTA (0.04% w/v), centrifuged at 2500 rpm for 15 min at 10 °C, and processed for low density lipoproteins (LDL) separation. LDL were isolated by sequential density-gradient ultracentrifugation, using a Beckman Optima XL90 ultracentrifuge (Palo Alto, CA, USA) equipped with a Beckman 50 Ti fixed-angle rotor. Plasma was adjusted to a density of 1.063 g/mL by adding solid KBr, and the ultracentrifugation was performed at 40,000 rpm for 30 h, at 6 °C. LDL fraction was removed by tube slicing, transferred to another tube and centrifuged by using the same conditions after a further density adjustment to 1.019 g/mL with KBr. The LDL-containing bottom layer (density 1.019–1.063 g/mL) was isolated by slicing the centrifuge tube. The LDL stock suspension was purged with nitrogen, dialyzed in a 100-fold volume of 0.01 M PBS pH 7.4 and stored for 24 h at 4 °C in the dark.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord of healthy donors and used at passage 2–6 as previously reported [10,11]. Samples were collected following the ethical guidelines of Helsinki declaration. Briefly, the serum-free PBS containing type II collagenase (10 mg/mL) was used to perfuse the umbilical vein in order to remove the endothelial cell layer. After dissociation, the cells were collected in flasks and cultured in endothelial cell growth medium (EGM-2 BulletKit, LONZA, Verviers, Belgium) supplemented with 10% FBS and maintained in a humidified atmosphere containing 5% CO_2, at 37 °C. The culture medium was replaced every 2–3 days; subcultures were performed with trypsin-EDTA.

2.3. Chemical reagents

2-Thiobarbituric acid (TBA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), baicalein, butylated hydroxytoluene (BHT), copper(II) sulphate ($CuSO_4$), dichlorofluorescin diacetate (DCFH-DA), 1,1-diphenyl-2-picrylhydrazyl (DPPH.), ethylene diaminetetraacetic acid disodium (Na_2-EDTA), eupatorin, fluorescein, galangin, magnolol, myricetin, oleuropein, potassium bromide (KBr), silibinin and N-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), isopropyl alcohol and HPLC grade methanol came from Carlo Erba (Milano, Italy). Dulbecco's modified Eagle medium (DMEM), endothelial cell basal medium (EBM-2) and endothelial cell growth medium (EGM-2), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin-EDTA were obtained from Lonza (Walkersville, MD, USA). 2,2′-Azobis-(2-amidinopropane) dihydrochloride was purchased from Acros Organics (Geel, Belgium), while type II collagenase from Clostridium histolyticum (250 u/mg) and trichloroacetic acid (TCA) were purchased from Worthington (Lakewood, NJ, USA) and Merck (Darmstadt, Germany), respectively.

2.4. Radical scavenging capacity assays

The DPPH radical scavenging assay was performed according to the method reported by Brand-Williams [12], with some modifications previously described [13]. Standard solutions or DMSO (control) were prepared and added to 70 μM DPPH methanolic solution; the mixtures were kept in the dark, for 60 min, and the absorbance was read at 517 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA, USA). Radical scavenging capacity was expressed as percentage effect (E %). The Oxygen Radical Absorbance Capacity (ORAC) assay was performed according to the method of Gillespie [14], with some modifications [15]. Briefly, 750 μL of 0.08 μM fluorescein was mixed with 125 μL of sample (5 μM) or PBS (blank) or trolox standard solution (6.25–50 μM). The plate was then incubated at 37 °C, for 10 min; the oxidative reaction was initiated by adding 125 μL of AAPH 0.15 M, and the fluorescence was recorded every 5 min for 60 min at 37 °C using a PerkinElmer Victor™ X3 microplate reader (Waltham, MA, USA). The ORAC values were expressed as TEAC (Trolox Equivalent Antioxidant Capacity, μmol TE/μmol tested compound).

2.5. TBARS assay

The extent of LDL oxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to the TBA test [16]. Briefly, 480 μL of human LDL suspension (25 μg protein/mL) in PBS and 24 μL of sample methanolic solutions (2.5 and 5 μM) were added into centrifuge tubes and left to stand for 15 min, at room temperature. Afterwards, the oxidation was started by 10 μM $CuSO_4$ 0.4 mM, for 1 h, at 37 °C. After that the tubes were carried on ice and were added 50 μL of 2 mM Na_2-EDTA, 25 μL of 2 g/L BHT, 250 μL of 100 g/L TCA and 500 μL of 6.7 g/L TBA. The tubes were purged with nitrogen, gently mixed and heated at 100 °C for 20 min. Afterwards, the tubes were centrifuged at 3000*g* for 5 min. The supernatant absorbance was measured at 532 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA, USA). Quantification of TBARS was performed by converting the absorbance in malondialdehyde equivalents through the relative molar extinction coefficient.

2.6. Human LDL oxidation kinetics

The oxidation of LDL was measured essentially as described by Esterbauer [17]. Briefly, 480 μL of LDL suspension (25 μg protein/mL) in PBS and 24 μL of sample methanolic solutions (0.5–3 μM) were added into cuvettes and left to stand for 15 min, at room temperature. Afterwards, the oxidation was initiated as described for the TBARS procedure. Absorbance was recorded every 5 min at 37 °C, and the oxidation kinetic was followed by measuring the increasing levels of conjugated dienes at 234 nm for up to 8 h. The degree of LDL oxidation was assessed using the Lagtime index, defined as the interval (min) between the intercept of the linear least-square slope of the curve with the initial-absorbance axis, and the inhibition rate (I.R. %), defined as:

$$\text{I.R.}\% = \frac{\text{lagtime}_{\text{sample}} - \text{lagtime}_{\text{ctrl}}}{\text{lagtime}_{\text{ctrl}}} \times 100$$

2.7. Cell viability

Cultured HUVEC were used from passage 2 to 6; the viability was determined using the MTT assay. The cells were treated with each phytoconstituent (0.01–10 μM) for 24 h in serum-free endothelial cell basal medium (EBM-2, Lonza, Verviers, Belgium).

After 24 h, cells were replaced with fresh medium added with 20 μL MTT (5 mg/mL) and incubated for 4 h at 37 °C. The cells were then lysed by replacing the medium with 200 μL of acidified isopropanol. The absorbance at 570 nm was determined using a Victor x3 multilabel counter, PerkinElmer (USA).

2.8. DCFH-DA cell-based assay

The effect of polyhydroxyl compounds on H_2O_2-induced oxidative stress in HUVEC was determined by a fluorimetric assay using DCFH-DA. In brief, after preincubation for 1 h with each compound (0.01–5 μM), HUVEC were incubated with 25 μM DCFH-DA for 30 min. The fluorescence intensity was measured at 485 nm excitation and 538 nm emission wavelengths using a PerkinElmer Victor™ X3 microplate reader (Waltham, MA, USA). After basal condition assessment, H_2O_2 was added to each well at the concentration of 500 μM, for 2 h at 37 °C, before the fluorescence reading.

2.9. Statistical analysis

Data are expressed as mean ± SEM of at least three experiments. Sigmoid curve fitting was performed using GraphPad Prism software 5 P [13]. Statistical comparisons were performed using ANOVA followed by Tukey multiple comparisons. The differences between control and treatment were assessed by Student's t test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Radical scavenging activities of polyhydroxyl compounds

The effects of baicalein, bilobalide, eupatorin, galangin, magnolol, myricetin, oleuropein and silibinin were firstly evaluated by means of DPPH and ORAC assays, using N-acetylcysteine (NAC) as reference compound. All compounds were tested from 0.1 μM to 10 mM using the DPPH assay. Most of them showed concentration-dependent effects, achieving the full radical inhibition at the highest concentrations, with the exception of 10 mM bilobalide, which reached only the 24% of total scavenging effect (Fig. 1A). On the basis of the pEC_{50} ($-\log EC_{50}$) values, myricetin showed the highest antioxidant capacity (5.36 ± 0.04, $p < 0.05$ *vs.* oleuropein), followed by oleuropein (4.91 ± 0.05, 0.05 *vs.* baicalein), baicalein (4.67 ± 0.05), galangin (4.51 ± 0.04, $p < 0.05$ *vs.* magnolol), magnolol (3.91 ± 0.03, $p < 0.05$ vs. eupatorin), while eupatorin (3.55 ± 0.06) and silibinin (3.53 ± 0.02) showed lower antioxidant capacities. The reference compound NAC, with a pEC_{50} of 4.76 ± 0.08, showed lower scavenging capacity of myricetin ($p < 0.05$) but similar to oleuropein and baicalein. Thus, the EC_{50} of myricetin is about 50-fold lower than eupatorin and silibinin, suggesting myricetin us the best anti-radical compound in this assay based on single electron transfer (SET) reaction [18]. Further, the ORAC assay, based on hydrogen atom transfer (HAT) reaction [19], was also used to evaluate the properties of the polyhydroxyl compounds (Fig. 1 B). Using this assay, oleuropein showed the highest antioxidant capacity (8.25 ± 0.25 TEAC), followed by myricetin (6.76 ± 0.41 TEAC), magnolol (5.00 ± 0.38 TEAC), silibinin (4.74 ± 0.22 TEAC), eupatorin (4.37 ± 0.60 TEAC), galangin (3.97 ± 0.22 TEAC), and baicalein (1.83 ± 0.21 TEAC).

Myricetin and oleuropein showed the highest antioxidant potential referring to both DPPH and ORAC assays, even higher than the antioxidant NAC. Conversely, bilobalide did not show any significant activity in both assays; for this, it was not further investigated.

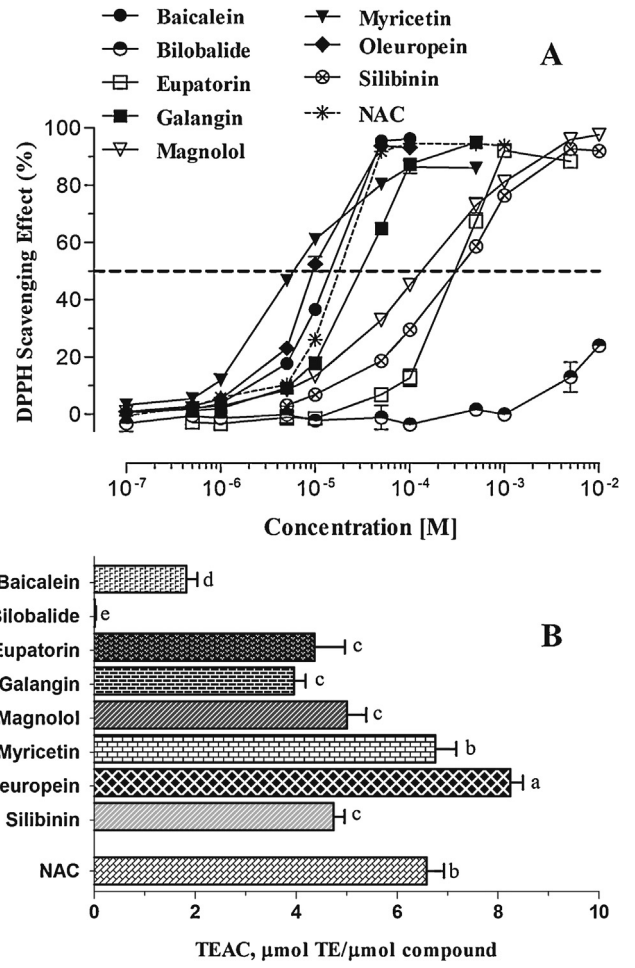


Fig. 1. Radical scavenging effect of 0.1–10 mM polyhydroxyl compounds determined by DPPH (A) and ORAC (B) assays. Undisclosed SEM fall within respective symbols on the graph. NAC: *N*-acetylcysteine. The bar values in the graph of ORAC assay (B) indicated by the same letter are not significant by the Tukey's multiple range test ($p < 0.05$). For the DPPH assay the significance of EC_{50} values of the compounds is reported in the results.

3.2. Effects of polyhydroxyl compounds on human LDL oxidation

The oxidation of LDL was determined by measuring the amount of malondialdehyde (MDA) as TBARS. We studied the capacity of the polyhydroxyl compounds to counteract TBARS production from LDL oxidation catalyzed by 10 μM $CuSO_4$ (Fig. 2). Oleuropein, myricetin and magnolol at 2.5 μM significantly decreased TBARS production of 85%, 65% and 48%, respectively (Fig. 2A), whereas the other compounds did not significantly change the MDA production (Fig. 2A). However, most of them at the higher concentration of 5.0 μM reduced the TBARS production, with the exception of galangin and silibinin (Fig. 2 B). The lipoprotein oxidation was further analyzed by monitoring the LDL oxidation kinetics in the presence of each polyhydroxyl compound using the Lagtime index (Table 2). In fact, the increase in absorbance at 234 nm reveals the production of conjugated dienes and indicates the susceptibility of LDL towards oxidation: a more prolonged lag phase means an increased resistance of LDL to oxidation and hence protection induced by the added compound. Myricetin and oleuropein at 1.0 μM showed the highest antioxidant activity with an inhibition rate around one hundred per cent (Table 2). Indeed, oleuropein and myricetin delayed the LDL oxidation time from about 3 to 6 h.

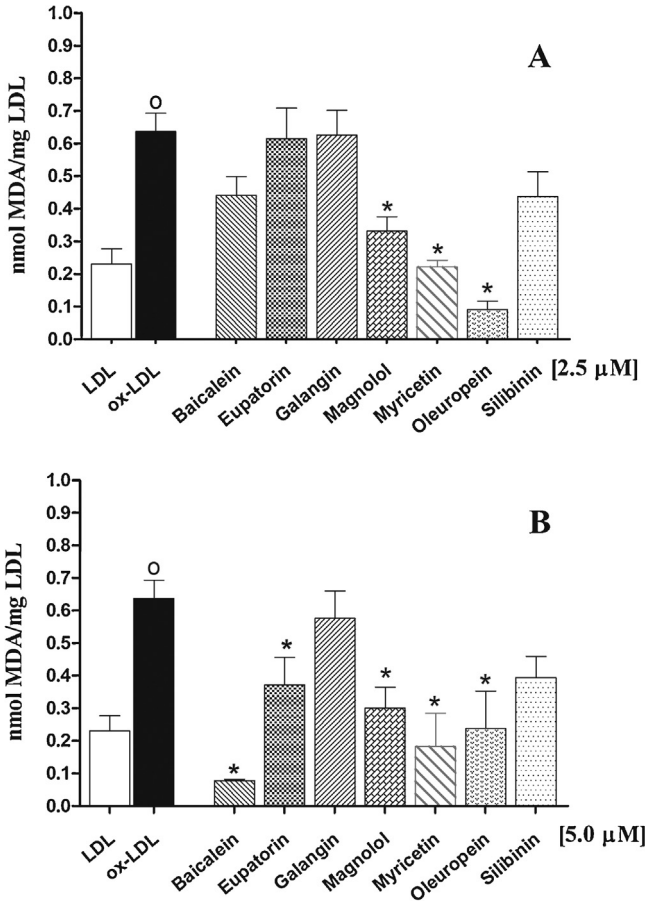


Fig. 2. Effects of polyhydroxyl compounds, 2.5 μM (A) and 5 μM (B), on copper-induced LDL oxidation using TBARS assay. MDA = malondialdehyde. °: $p < 0.05$ ox-LDL *vs.* LDL; *: $p < 0.05$ treatment *vs.* ox-LDL.

Magnolol (1.0 μM) and baicalein (1.5 μM) also increased the Lagtime of 49.5 and 42.6%, respectively; while galangin showed an inhibition rate of 15.5% at 2.0 μM and 58.0% at 3.0 μM.

3.3. Effects of polyhydroxyl compounds on H_2O_2-induced oxidative stress in cultured HUVEC

Finally, we studied the activities of the compounds in HUVEC to investigate the endothelial oxidative stress. Preliminarily, we performed the MTT assay to determine whether the compounds could have any direct cytotoxic effects. None of them showed any influence on HUVEC viability up to 10 μM. Further, H_2O_2-induced oxidative stress was detected using the DCFH-DA assay. Among the polyhydroxyl compounds, pre-incubated for 1 h before treatment with 500 μM H_2O_2 (2 h), myricetin showed protective effects at all concentrations tested (Fig. 3). While galangin showed antioxidant activity only at the higher concentration of 5 μM. Unexpectedly, none of the other polyhydroxyl compounds were able to reduce significantly the H_2O_2-induced oxidative stress.

4. Discussion

Epidemiological studies have suggested that the consumption of foods and beverages rich in flavonoid and phenolic compounds correlates with lower risk of various diseases, including cardiovascular diseases. Today, moreover, a large amount of food supplements are available around the world with few evidence of their effectiveness. In this context, it is important to compare the activities of different health plant-derived compounds to find the most promising compounds through suitable *in vitro* assays before defining appropriate clinical trials. In the present research several polyhydroxyl compounds, such as baicalein, bilobalide, eupatorin, galangin, magnolol, myricetin, oleuropein and silibinin were studied against oxidative stress *in vitro*. All the compounds were able to reduce the DPPH radical, showing concentration-dependent scavenging activity, with the exception of bilobalide, a typical constituent of *Ginkgo biloba* leaves. Myricetin showed the highest radical-scavenging capacity, followed by oleuropein, baicalein, galangin, and then the others. In agreement with these results, the higher ORAC values were found for oleuropein and myricetin, while magnolol, silibinin, eupatorin, galangin, and baicalein showed progressively decreasing activities. Bilobalide still was the worst since did not show any scavenging activity. However, in literature, *Ginkgo biloba* leaf extracts are reported having antioxidant properties [20]. But generally these activities are related to the presence of flavonoids, proanthocyanidins and catechins, whereas the sesquiterpene bilobalide seems to have low antioxidant capacity [21].

It is known that only oxidatively modified LDL are recognized by scavenger receptors causing the transformation of macrophages into foam cells, which are strongly implicated in atherosclerotic plaque formation. In addition, ox-LDL are able to activate various transcription factors (*e.g.* NF-kB) in endothelial cells, macrophages and smooth muscle cells, also inducing expression of genes encoding adhesion molecules, cytokines and growth factors, amplifying the inflammatory response [22].Therefore, the inhibition of LDL oxidation could be an important target against

Table 2
Lagtime values and relating inhibition rate of the polyhydroxyl compounds, obtained from the copper-induced LDL oxidation kinetics.

Compound	Concentration [μM]	Lagtime[1] [min]	Lagtime[2] [min]	Inhibition rate [%]
Baicalein	1.0	106 ± 5	123 ± 7	16.4
	1.5		150 ± 27	42.6
Eupatorin	1.0	172 ± 71	n.d.	n.d.
	1.5		n.d.	n.d.
Galangin	2.0	111 ± 28	127 ± 22	15.5
	3.0		177 ± 53*	58.0
Magnolol	0.5	183 ± 21	257 ± 66	39.2
	1.0		275 ± 61*	49.5
Myricetin	0.5	176 ± 37	201 ± 50	13.6
	1.0		351 ± 82*	98.9
Oleuropein	0.5	183 ± 33	221 ± 18*	22.3
	1.0		374 ± 78*	104.4
Silibinin	1.0	212 ± 97	n.d.	n.d.
	1.5		n.d.	n.d.

Lagtime[1]; basal values. Lagtime[2]; values in the presence of each compound. *; $p < 0.05$ *vs.* Lagtime[1]. Each concentration was chosen on the basis of pilot experiments starting from 0.5 μM up to 5 μM. n.d.; value not determinable (for eupatorin and silibinin the extrapolation of the Lagtime was not reliable because the kinetic curves lost their typical sigmoid profile).

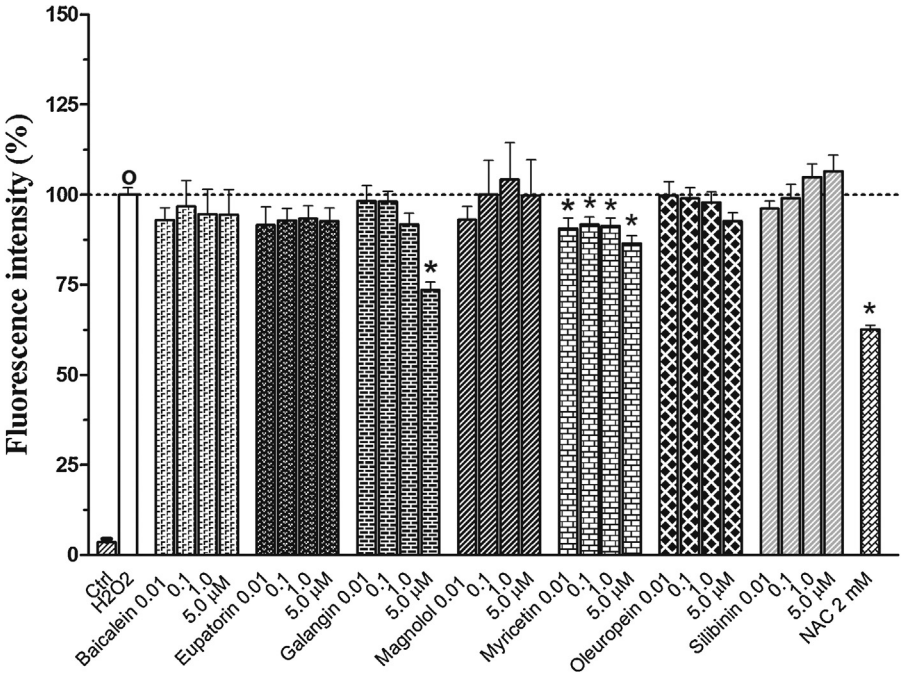


Fig. 3. Effects of polyhydroxyl compounds on H_2O_2-induced oxidative stress in cultured HUVEC detected by DCFH-DA assay. The human cells were pre-incubated with each compound for 1 h and treated with 500 μM H_2O_2 for 2 h. *: $p < 0.05$ treatment *vs.* control. NAC: *N*-acetylcysteine.

atherosclerotic diseases. Among the compounds studied, oleuropein, myricetin and magnolol already at 2.5 μM significantly decreased TBARS production showing higher activity than baicalein, eupatorin, galangin, and silibinin. Moreover, 1.0 μM myricetin and oleuropein strongly delayed the oxidation kinetics of LDL, lengthening the Lagtime.

An overview on the activities of myricetin and the other polyhydroxyl compounds is reported in Table 3 where a qualitative score for each phytoconstituent, according to the outcomes obtained in each assay, was given for a comprehensive description of their activities. To facilitate the assessment, the compounds are listed in decreasing order of activity; it is clear that myricetin is the substance which has shown a better performance, followed by oleuropein, galangin, baicalein, and magnolol, whereas both eupatorin and silibinin exhibit slight activities.

Recently, Qin and collaborators reported that myricetin 3-*O*-rhamnoside (myricitrin) inhibited copper-mediated LDL-oxidation and ROS production in endothelial cells upon ox-LDL treatment [23]. Furthermore, the deglycosylation of myricitrin into myricetin by human intestinal microbiota has been recently reported [24], suggesting similar effectiveness of oral myricitrin or myricetin administration. In the present research, myricetin showed a persistent protection against H_2O_2-induced stress in cultured HUVEC, which is a very drastic oxidation condition. This observation is in agreement with literature data and is of clinical interest since the amount of myricetin required as antioxidant is even lower than micromolar concentrations and reachable by oral administration [25]. Concerning safety, intraperitoneal injection in mice of myricetin 1000 mg/kg body weight did not reveal any toxic effects or fatalities [26], and its use is recognized safe as ingredient of fruits and vegetables suitable for human consumption.

5. Conclusions

Taken together, the above results indicate myricetin as the most active polyhydroxyl compound among the eight plant-derived compounds studied, inhibiting free radicals, LDL oxidation and ROS formation in cultured HUVEC. Thus myricetin can constitute a

Table 3
Overall estimation of the antioxidant activity of the polyhydroxyl compounds obtained from the assays performed.

Compound	DPPH	ORAC	TBARS	LDL oxidation kinetics	HUVEC oxidative stress
Myricetin	+++	+++	+++	+++	++
Oleuropein	++	+++	+++	+++	+/−
Galangin	++	++	+/−	+	+
Baicalein	++	+	++	++	+/−
Magnolol	+	++	+++	++	+/−
Eupatorin	+	++	++	−	+/−
Silibinin	+	++	+/−	−	+/−

A specific score was assigned on the basis of the activity detected by each assay. +++: excellent; ++: good; +: weak; +/−: not relevant; −: not detected. Bilobalide was not inserted because did not show any significant activity (see Section 3).

possible pharmacological agent against oxidative stress-related disorders, as atherosclerosis. Overall, current data encourage planning of clinical trials on myricetin against oxidative stress-related cardiovascular pathologies.

Conflict of interest

The authors declare that there is no competing interest.

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