Bioorganic & Medicinal Chemistry 17 (2009) 6173–6179

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Antioxidant efficacy of iridoid and phenylethanoid glycosides from the medicinal plant *Teucrium chamaedris* in cell-free systems

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ARTICLE INFO

Article history:
Received 12 March 2009
Revised 24 July 2009
Accepted 26 July 2009
Available online 30 July 2009

Keywords:
Teucrium chamaedrys
Iridoid glycosides
Phenylethanoid glycosides
NMR analysis
Antioxidant activity
Cell-free systems

ABSTRACT

Twelve glycosides, seven iridoids and five phenylethanoids, have been isolated from leaf and root methanolic extracts of Wall Germander (*Teucrium chamaedrys*), a Mediterranean species historically used as a medicinal plant. Among them, three iridoid and one phenylethanoid glycosides have been isolated and characterized for the first time. All of the structures have been elucidated on the basis of their spectral data, especially 1D and 2D NMR experiments.

The antioxidative properties of pure metabolites, as well as of crude organic extracts of the plant, have been analyzed on the basis of their DPPH radical scavenging capability. The antioxidant capacity in cell-free systems of the isolated metabolites was carried out by measuring their capabilities to inhibit the synthesis of thiobarbituric acid reactive species in assay media using as oxidable substrates a vegetable fat and the pentose sugar 2-deoxyribose and to prevent oxidative damage of the hydrosoluble bovine serum albumin (BSA) protein. Phenylethanoid glycosides resulted efficacious DPPH radical, while iridoid glycosides prevent massively the 2-deoxyribose and BSA oxidations in assay media.

1. Introduction

Many medicinal plants have aroused interest as sources of natural bioactive products and some of them have screened for their potential uses as remedies for the treatment of many diseases and preservation of food from oxidant deterioration. Their preservative effect is due to the presence of antioxidative constituents able to delay or inhibit the oxidation of lipids or other biomolecules by inhibiting the initiation and/or propagation of oxidative chain reactions. The oxidative damage has an important etiological role in aging and in the development of diseases as cancer, atherosclerosis and other inflammatory disorders. It is known that a lower incidence of these pathologies occur in people eating larger amounts of antioxidant-containing fruits and vegetables.[1] Medicinal plants contain a wide variety of natural antioxidants such as phenolic acids, flavonoids and tannins, which possess more potent antioxidant activity than common dietary plants. In the last years, the search of antioxidant molecules from natural sources has come to the forefront of modern research and, recently, we reported the isolation of antioxidant metabolites from edible and medicinal plants growing in Mediterranean area.[2–7]

Among Mediterranean plants, those belonging to the Lamiaceae family, such as sage, oregano and thyme, have showed strong antioxidant activity.[8]

The Mediterranean shrub known as *Teucrium chamaedrys* (Germander) has been used, in the past, as medicinal plant for its anti-inflammatory, anti-rheumatic, digestive and diuretic effects.[9] It is used externally as an astringent infusion on the gums and also in the treatment of wounds. *T. chamaedrys*, which is one of the most common and highly investigated species in the *Teucrium* genus, is marketed for use in weight control, and as additive for liquors, although there have been some concerns over hepatotoxicity due to its content in *neo*-clerodane diterpenes.[10] In fact, the hepatotoxicity of germander and that of one of its major furanoneoclerodane diterpenes, teucrin A, were investigated in mice. Teucrin A was found to cause the same midzonal hepatic necrosis as observed with extracts of the powdered plant material.[11]

In this paper we reported the evaluation of radical scavenging activity of crude extracts of *T. chamaedrys* (L.), the isolation and characterization of iridoid and phenylethanoid glycosides from methanolic extracts and the assessment of radical scavenging and antioxidant efficacies in cell-free systems.

2. Results and discussion

A previous *screening* turned to the radical scavenging efficacy determination, was carried out on leaf and root crude extracts from

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doi:10.1016/j.bmc.2009.07.065

T. chamaedrys. The evaluation of antioxidant capacity was performed undergoing each extract to DPPH radical antioxidative HAT (Hydrogen Atom Transfer) assay (Fig. 1). The oxidant 2,2′-diphenyl-1-picrylhydrazyl (DPPH), characterized as a stable nitrogen radical by virtue of the delocalization of the unpaired electron over the molecule as a whole, acts as probe for monitoring the reaction and as an indicator of the reaction endpoint.

The DPPH radical scavenging capacity determination showed peculiar efficacy of leaf and root methanolic extracts. The highest antiradical effect was realized in complete conversion of the radical to the corresponding reduced form, by 83.0 μg/mL sample dose. ID_{50} (*Inhibition Dose*) values, defined as the dose of extract that causes 50% loss of the DPPH capacity, are obtained from the plotted graph of scavenging activity against substrate doses (Table 1). Root methanolic extract showed an ID_{50} value of 6.18 μg/mL. The strong antiradical efficacy became significant when compared with the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, also known as Trolox®, an antioxidant water-soluble synthetic derivative of vitamin E, commonly used as standard (ID_{50} 5.3 μg/mL).[12] Analogously the most polar leaf extract had a marked radical scavenger effect (7.13 μg/mL).

The bioactive methanolic crude extracts were submitted to extractive and chromatographic analyses to give 12 compounds: seven iridoid and five phenylethanoid glycosides, four of them isolated and characterized for the first time.

The new compound **1** (Fig. 2) showed, in the downfield region of ^{1}H NMR spectrum (Table 2), two coupled doublets at δ 7.59 and 6.37, three aromatic protons of a 1,2,4-trisubstituted benzene ring at δ 7.16, 6.96 and 7.20, as well as two further proton signals at δ 6.23 and 5.95. In the carbinolic region, an anomeric proton at δ 4.68, a diasterotopic methylene as two doublet of doublets at δ 3.94 and 3.71, besides three overlapped protons ranging from 3.20 to 3.40 ppm, were present, suggesting the presence of a monosaccharide moiety in the molecule. The downfield shift of the values of the H-6 doublet of doublets allowed to hypothesize the presence of an acyl group on the primary alcohol group of the sugar. The remaining signals in the ^{1}H NMR spectrum were a doublet of doublet at δ 4.73, a carbinol methine at δ 4.06, two overlapped methoxyls at δ 3.86, two methines at δ 2.95 and 2.84, a second diasterotopic methylenes at δ 2.28 and 2.16, and finally, a methyl as singlet at δ 1.60. The ^{13}C NMR spectrum showed 25 peaks, one of them due to the magnetically equivalent methoxyls, identified, by a DEPT experiment, as two methyl signals, two methylenes, 16 methines and five tetrasubstituted carbons. The HSQC-TOCSY experiment allowed to identify the sugar moiety. In fact their H-1 and H-6 protons correlated with the carbons at δ 100.2 (anomeric), 74.8, 78.1, 71.8, 78.2 and 63.1. The COSY and HMBC experiments (Fig. 4) let us individuate the carbon sequence of the sugar, whose δ values were in good accordance with those of

Table 1
DPPH radical scavenging capacity of *T. chamaedrys* organic extracts reported as ID_{50} values and TEAC (Trolox® equivalent antioxidant capacity)

Leaf extract	ID_{50} (μg/mL)	TEAC	Root extract	ID_{50} (μg/mL)	TEAC
MeOH	7.13	0.74	MeOH	6.18	0.86
EtOAc	25.05	0.21	EtOAc	77.80	0.07
PE	>1000	n.c.	PE	253.30	0.02

n.c. = not calculated. Trolox® ID_{50} = 5.3 μg/mL. PE = Petroleum ether extract, EtOAc = ethyl acetate extract; MeOH = methanolic extract.

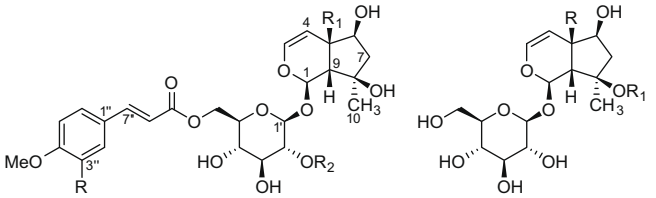


Figure 2. Iridoid glycosides from *T. chamaedrys*.

a glucopyranose. The β configuration of the anomeric carbon was attributed on the basis of the coupling constant value of the anomeric proton. The H-6 protons of glucose showed, in the HMBC spectrum, correlations with an estereal carbon at δ 169.1, which correlated, in turn, with both the doublets at δ 6.37 and 7.59. This latter correlated with the aromatic carbons at δ 128.9, 123.9 and 111.5, suggesting the presence of a cinnamoyl moiety in the molecule. The correlation of the methoxyl protons with the carbons at δ 152.8 and 150.8 indicated the presence of a 3,4-dimethoxycinnamic acid esterified to the 6-OH of the sugar. The anomeric carbon was heterocorrelated with the proton at δ 5.95, bound to the carbon at δ 94.8, which was, in turn correlated to the anomeric proton. The carbon at δ 94.8 was correlated to the protons at 2.95, 2.84 and 6.23. This latter showed cross peaks with the proton at δ 4.73, in the COSY, which was correlated to the proton at δ 2.84. This proton resulted correlated, besides to the signal at δ 2.95, to the proton at δ 4.06, which correlated to the methylene protons at δ 2.16 and 2.28 bound to a carbon at δ 47.8. Finally, the HMBC experiment showed correlations between the carbon at δ 47.8 and the protons at δ 2.28, 4.06 and 1.60, and between the tertiary carbinol carbon at δ 79.5 and the protons at δ 2.95, 2.84, 4.06, 2.16, 2.28 and 1.60. These data indicated the presence of a nor-iridoid bringing three hydroxyl groups to the C-1, C-6 and C-8 carbons.

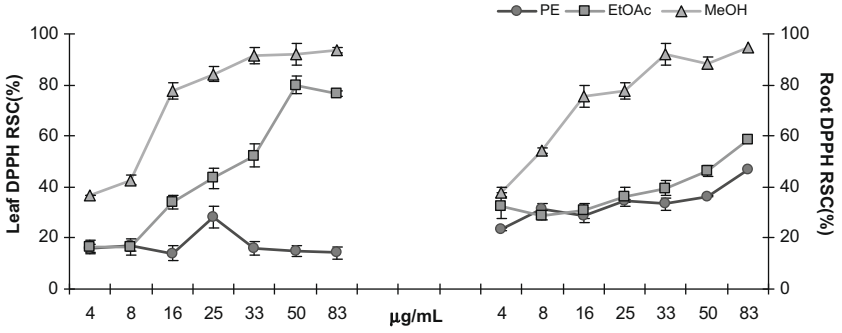


Figure 1. DPPH radical scavenging capacity (RSC, %) of *T. chamaedrys* organic extracts. Values are reported as percentage versus a blank ± SD. PE = Petroleum ether extract, EtOAc = ethyl acetate extract; MeOH = methanolic extract.

Table 2
^{1}H and ^{13}C NMR data of the new iridoids glycosides **1–3** from *T. chamaedrys* in CD_3OD

	1		**2**		**3**	
	δ^1H	δ^{13}C	δ^1H	δ^{13}C	δ^1H	δ^{13}C
1	5.95 d (1.5)	94.8	6.18 s	94.7	6.26 s	94.3
3	6.23 dd (6.3, 1.5)	141.3	6.41 d (6.3)	143.9	6.37 d (6.3)	143.4
4	4.73 dd (6.3, 1.5)	104.2	4.93 dd (6.3, 1.8)	106.9	4.95 dd (6.3, 1.5)	107.7
5	2.84 br d (8.4)	41.8	–	73.4	–	74.2
6	4.06 d (4.5)	77.0	3.75 ov	77.7	3.82 ov	77.4
7	2.28 d (14.7)	47.8	2.26 d (15.1)	46.3	2.27 d (15.6)	46.6
	2.16 dd (14.7, 3.0)		2.02 dd (15.1, 4.5)		1.96 dd (15.6, 4.5)	
8	–	79.5	–	88.5	–	88.5
9	2.95 d (8.1)	49.6	2.93 s	55.7	2.97 s	54.8
10	1.60 s	23.0	1.52 s	22.7	1.51 s	22.8
1′	4.68 d (7.8)	100.2	4.62 d (8.1)	100.0	4.71 d (7.8)	96.2
2′	3.21 t (9.0)	74.8	3.22 t (8.9)	74.7	3.58 ov	80.3
3′	3.33 ov	78.1	3.34 ov	78.1	3.35 ov	78.0
4′	3.30 ov	71.8	3.30 ov	71.8	3.35 ov	71.1
5′	3.33 ov	78.2	3.33 ov	78.2	3.35 ov	78.6
6′	3.94 dd (11.1, 2.4)	63.1	3.92 dd (11.1, 1.8)	63.0	3.90 dd (12.6, 2.1)	62.7
	3.71 dd (11.1, 5.7)		3.73 dd (11.1, 5.7)		3.71 dd (12.6, 4.8)	
1″	–	128.9	–	129.2	–	128.4
2″	7.20 d (1.8)	111.5	7.20 d (2.1)	124.0	7.54 d (8.7)	130.9
3″	–	150.8	–	112.7	6.95 d (8.7)	115.4
4″	–	152.8	–	152.9	–	163.2
5″	6.96 d (8.7)	112.7	6.97 d (8.4)	150.4	6.95 d (8.7)	115.4
6″	7.16 dd (8.7, 1.8)	123.9	7.16 dd (8.4, 2.1)	111.5	7.16 d (8.7)	130.9
7″	6.37 d (15.9)	118.0	6.38 d (15.9)	117.8	6.34 d (15.9)	117.4
8″	7.59 d (15.9)	146.0	7.60 d (15.9)	146.2	7.62 d (15.9)	146.0
9″	–	169.1	–	169.1	–	169.2
OMe	3.86 s	56.0	3.86 s	56.5	3.83 s	55.9
OMe	3.86 s	56.0	3.86 s	56.5	–	–
1‴					4.75 d (7.8)	104.4
2‴					3.21 t (8.9)	71.2
3‴					3.33 ov	78.4
4‴					3.30 ov	71.1
5‴					3.33 ov	78.5
6‴					3.82 ov	62.1
					3.65 ov	

br d = broad doublet, d = doublet, dd = doublet of doublet, ov = overlapped, s = singlet t = triplet; the *J* values are reported in the brackets.

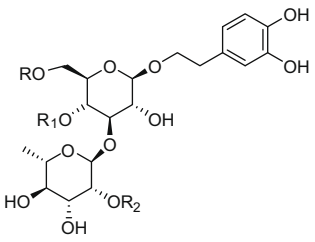
The ^{1}H NMR spectrum of compound **2** (Table 2) showed the same 3,4-dimethoxycinnamoyl moiety as two olefinic protons at δ 7.60 and 6.38, three aromatics at δ 7.20, 7.16 and 6.97 and two coincident methoxyls at δ 3.86. The sugar signals indicated the presence of a glucose acylated to the C-6 carbon. The main differences regarded the iridoid moiety. In fact, the H-1, H-2 and H-3 protons resonated at δ 6.18, 6.41 and 4.93, respectively. In the upfield region of the spectrum were present the two doublet of doublet at δ 2.26 and 2.02, the H-10 methyl at δ 1.52 and a methine as singlet at δ 2.93. The ^{13}C NMR showed the presence of 25 signals, identified as two methyls, two methylenes, 15 methines and six tetrasubstituted carbons. The singlet at δ 2.93 resulted bound to the carbon at δ 55.7, in the HSQC experiment, and showed long range correlations, in a HMBC, with the C-1, C-4, C-5, C-7 and C-8; in turn, the carbon at δ 55.7 showed cross peaks with the H-1, H-4, H-6, H-7 and H-10 protons. These data suggested the presence of a nor-iridoid bringing four hydroxyls bound to the C-1, C-5, C-6 and C-8 carbons. The heterocorrelations between the anomeric carbon and the H-1 proton of terpene confirmed the same glycosylation point of the previous compound, while the value of the coupling constant of the anomeric proton confirmed the presence of a β-glucopyranoside.

Compound **3** (Fig. 2) was characterized by the presence of a disaccharide moiety in the molecule (Table 2). The 1D and 2D NMR data were in good agreement with the presence of the same iridoid of compound **2**. In the downfield region of ^{1}H NMR were evident the two protons of a trans double bond as doublets at δ 7.62 and 6.34, two doublets of an AA′BB′ spin system at δ 7.54 and 6.95, besides the H-1 and H-3 protons of the iridoid at δ 6.26 and 6.37, respectively. In the glycidic region of the spectrum, two anomeric signals were evident as two doublets at δ 4.75 and 4.71. The first signal bound with the carbon at δ 96.2, which showed, in the HMBC spectrum, correlations with the H-1 proton of aglycone. The HSQC-TOCSY experiment separated the overlapped ^{1}H spin systems and allowed the carbons assignment of the sugar moieties. The anomeric H-1‴ proton at δ 4.75, correlated, in the COSY, to the H-2″ proton at δ 3.58. This latter, correlated to the carbon at δ 80.3 in the HSQC, showed cross peaks with the anomeric C-1‴ carbon at δ 104.4. Furthermore, the H-6′ protons were heterocorrelated to the carboxyl carbon at δ 169.2, confirming the acylation position. Finally, both the anomeric carbons showed β configurations, as indicated by the *J* values in the ^{1}H NMR spectrum.

Compound **4** was already reported as neuroprotective agent, isolated from *Scrophularia buergeriana* roots.[13] Compounds **5** and **6** lacked of the cinnamoyl moiety at the C-6′ carbon of glucose and shared the same acetyl group esterified at the hydroxyl group bound at the C-8 carbon of aglycone. Both the compounds have been isolated from *Physostegia virginiana* L.[14] Finally, compound **7**, the last iridoid isolated in *T. chamaedrys* was identified as harpagide, already reported from the medicinal plant *Harpagophytum procumbens* (devil's claw).[15]

Phenylethanoids **8** and **10** have been isolated from *Leucoseptrum japonicum*,[16] while compounds **9** and **11** have been previously reported as constituents of *T. chamaedrys*.[17]

Compound **12** (Fig. 3) was isolated and characterized for the first time. The ^{1}H NMR spectrum showed the signals of the hydroxytyrosol moiety as three aromatic protons at δ 6.66, 6.62



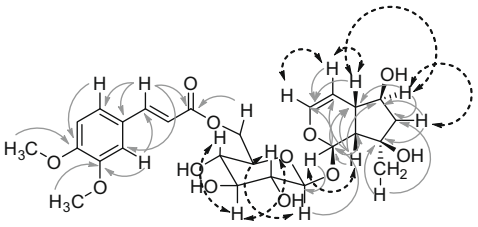
	R	R_1	R_2
8	H	caffeoyl	H
9	H	caffeoyl	α-L-Lyx
10	caffeoyl	H	α-L-Lyx
11	H	feruloyl	α-L-Lyx
12	feruloyl	H	α-L-Lyx

Figure 3. Phenylethanoid glycosides from *T. chamaedrys*.

and 6.52, a methylene triplet at δ 2.78 (H-7), and a diasterotopic methylene at δ 3.97 and 3.75 (H-8). The ^{1}H NMR data let identify the acylic moiety as a feruloyl group. In fact, besides two olefinic protons at δ 7.62 and 6.38, and aromatic protons at δ 7.15, 7.02 and 6.79, methoxyl protons as singlet at δ 3.86 was evident. These latter correlated, in the HMBC experiment, to C-3′ aromatic carbon at δ 147.1. The presence of three anomeric signals, in the ^{1}H NMR spectrum, suggested the presence of a trisaccharidic moiety in the molecule. The HSQC-TOCSY experiment allowed to assign all the ^{1}H and ^{13}C chemical shifts of the glycidic moiety. The identification of the sugars was confirmed by HPLC analysis of an hydrolyzed aliquot of compound **12**, obtained by treatment with 2 M TFA. The acylation position was hyphotized by the down shift of H-6 protons of glucose in the ^{1}H NMR spectrum and confirmed by 2D heteronuclear experiments.

The DPPH radical scavenging and antioxidant capabilities of the purified metabolites were assessed. Compounds from *T. chamaedrys* were tested in increasing concentration (5.0 μM, 10.0 μM and 20.0 μM) in triplicate analysis. The detected activities were compared to those exercised from Trolox®.

When DPPH radical scavenging was tested, phenylethanoid glycosides highly reduced the oxidant probe employing an activity strongly dose-dependent (Fig. 5). The comparison of relative IC_{50} values and Trolox® equivalents emphasizes molecules singular reducing efficacy (Table 3). Iridoid glycosides did not exhibit a good radical scavenging capacity at concentrations from 5 to 20 μM. The moderate radical scavenging capacity is probably due to their poor hydrogen-donating ability.



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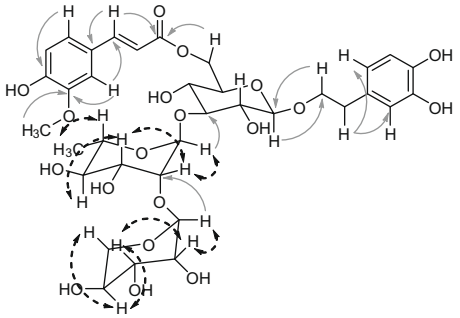

12

Figure 4. Selected HMBC (plain gray arrows) and COSY/TOCSY (dashed black arrows) correlations of iridoid glucoside **1** and phenylethanoid glycoside **12**.

The amount of metabolite **8** that is necessary to reduce by 50% the initial concentration of DPPH radical, as defined by the logarithmic regression curve of the graph scavenging capacity depending on the tested concentration range, was equal to 15.3 μM. Strong antioxidative capacity was exerted by the metabolite **11**, which C_6C_3 component was characterized by the presence of a feruloylic moiety in place of caffeoylic one present in compound **9**. Likewise, among the isolated metabolite, structurally different to the glycosylation site of C_6C_3, the metabolite **10** is relatively less active than its feruloylic derivative (**12**).

The definition of metabolites **2**–**12** antioxidant capacity was carried out through analysis of the thiobarbituric acid reactive species synthesis (TBARS) under oxidative stress conditions using as oxidizable substrate a vegetable fat (olive oil) and the pentose sugar 2-deoxyribose. The inhibiting capacity of isolated metabolites the protein oxidation, defined as the covalent modification of a protein induced either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress, was also measured (Fig. 6, Table 4). It is known that the peroxidative processes, which commonly place in living organisms, have their target in the macromolecules of biological interest. The peroxidation of organic substrates results in the formation of a complex variety of degradation products. The analysis of species produced or consumed during the peroxidative process, therefore, represents a method for quantitative determination of the peroxidative event itself.

The sensitivity of measuring thiobarbituric acid reactive substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. The unsaturated hydroxyaldehydes, lipoperoxidation secondary products, are able to react with the thiobarbituric acid at high temperatures resulting in typical pink/red products that have a maximum absorption at 532 nm. Metabolites, added in the reaction mixture during induction of UV light and high temperature mediated oxidative stress, were effective antioxidant agents the vegetable fat used as oxidation substrate. The investigated molecules showed an inhibiting lipoperoxidation capacity comparable or superior, in terms of IC_{50}, to that shown by Trolox®. Metabolite **8** was responsible for a peculiar efficacy, its low IC_{50} value (9.2) reflects its extraordinary capacity to contrast lipoperoxidative event. Although iridoid glycosides were less active than phenylethanoid ones, their IC_{50} value suggest their capabilities to act as antioxidants. Among them, metabolites **2** and **3** showed an antilipoperoxidative capacity comparable to that exercised by the used standard. The interaction of iron ions with hydrogen peroxide in biological systems can lead to the formation of a highly reactive tissue-damaging species that is thought to be the hydroxyl radical. The pentose sugar 2-deoxyribose is attacked by OH· radicals to yield a mixture of products. On heating with thiobarbituric acid at low pH, some or all of these products react to form a pink chromogen that can be measured by its absorbance at 532 nm. 2-Deoxyribose oxidation was induced by triggering the Fenton reaction, in which Fe^{2+} ions reacting with the pro-oxidant hydrogen peroxide formed Fe^{3+} ions, hydroxyl radicals and hydroxyl anions. Iridoid glycosides **2**–**4** were effective compounds. In particular metabolites **3** and **4** showed an IC_{50} value equal to 12.9 μM and 13.2 μM, respectively; corresponding Trolox® equivalent antioxidant capacity (TEAC), value (1.4) emphasizes its antioxidant capability. The presence of a further hydroxyl function on C-5 of iridoid skeleton and a 4″-methoxyphenylprop-7″-enoyl substituent on sugar moiety increases massively the antioxidant ability.

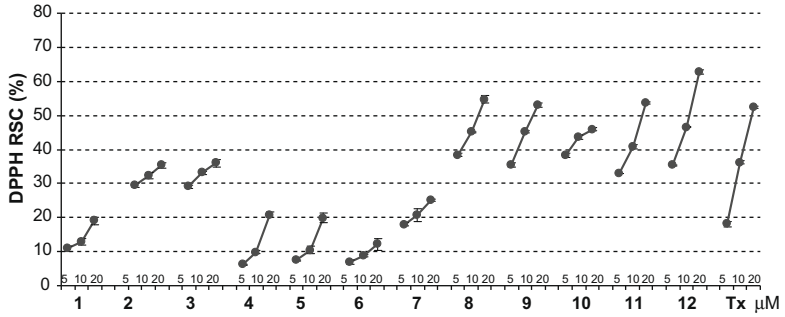

Figure 5. DPPH radical scavenging capacity (RSC, %) of *T. chamaedrys* metabolites **2**–**12**. Values are reported as percentage versus a blank ± SD. Tx = Trolox®.

Table 3
DPPH radical scavenging capacity of *T. chamaedrys* metabolites **1**–**12**

Compound	IC_{50}	TEAC
1	>300	n.c.
2	>300	n.c.
3	>300	n.c.
4	>300	n.c.
5	>300	n.c.
6	>300	n.c.
7	>300	n.c.
8	15.3	1.3
9	16.5	1.4
10	38.3	0.5
11	14.0	1.5
12	11.0	1.9
Trolox®	21.0	

Values as reported as IC_{50} (μM) and TEAC (Trolox® equivalent antioxidant capacity). n.c. = not calculated.

Table 4
Antioxidant capability of *T. chamaedrys* metabolites **2**–**12** on olive oil, 2-deoxyribose, bovine serum albumin (BSA)

Compound	IC_{50} Olive oil	TEAC	IC_{50} 2-Deoxyribose	TEAC	IC_{50} BSA	TEAC
2	33.1	1.0	26.5	0.7	10.7	6.5
3	39.0	0.8	12.9	1.4	12.8	5.4
4	67.3	0.5	13.2	1.4	10.4	6.6
5	44.2	0.7	52.2	0.3	38.9	1.8
6	45.5	0.7	51.0	0.3	23.5	2.9
7	44.1	0.7	36.8	0.5	41.1	1.7
8	9.7	3.3	26.4	0.5	57.2	1.2
9	16.3	2.0	34.6	0.5	44.8	1.5
10	26.3	1.2	33.5	0.7	38.0	1.8
11	20.9	1.5	32.0	0.6	32.8	2.1
12	46.7	0.7	37.3	0.5	18.1	3.8
Trolox®	31.8		18.0		69.2	

Values as reported as IC_{50} (μM) and TEAC (Trolox® equivalent antioxidant capacity).

Analogously phenyethanoid glycoside **9** was responsible for a marked activity, it was able to scavenge hydroxyl radicals by 54.7% at 20 μM concentration.

The hydroxyl radical is the oxygen radical with greater toxicity and reactivity: it lacks any endogenous mechanism of inactivation and it is the agent responsible for the initial phase of peroxide processes that occur at the level of body tissues; it is able, in fact, to damage the cellular macromolecules. The interaction of molecules with hydroxyl radical causes fragmentation of the protein in aminoacids components and increases levels of protein carbonyls and oxidation of free hydroxyl and thiolic functions. All of the metabolites induced a marked decrease in levels of protein carbonyls versus blank significantly higher than that determined by the Trolox®. Peculiar activity was exercised by iridoid glycosides **2**–**4** and phenylethanoid glycoside **12**. Iridoid glycoside **6** showed equally a good antioxidant activity confirming that the presence of the hydroxyl function on C-5 could increase antioxidant power of iridoid molecules.

The results show that oxidation and radical processes are highly complex and involve various mechanisms and targets. In particular the ability to scavenge free radical does not necessarily confer antioxidant properties. Phenylethanoid glycosides, in particular those possessing catechol moieties, showed strong antioxidant properties. Several studies suggest the role pharmacological of phenylethanoids. Lee et al.[18] have shown that phenylethanoid glycosides from leaves of *Callicarpa dichotoma* significantly attenuate neurotoxicity induced by the muscarinic antagonists glutamate and scopolamina, resulting in a considerable increase in cognitive ability in rats with a pronounced memory deficit. The molecules seem to be also able to inhibit the growth and proliferation of various cancer cells and to induce a reversible transformation of the same. In vitro studies demonstrate the effectiveness of these natural sub-

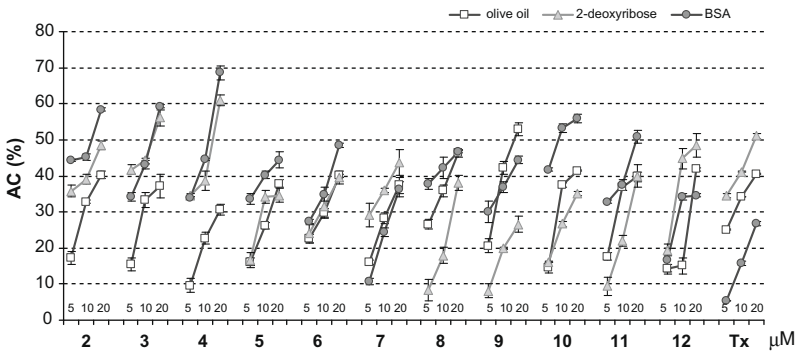

Figure 6. Antioxidant capability of *T. chamaedrys* metabolites **2**–**12** on (A) olive oil, (B) 2-deoxyribose, (C) bovine serum albumin (BSA). Values are reported as percentage versus a blank ± SD. Tx = Trolox®.

stances in determining the telomerase inhibition and the decrease of the length of cancer cells telomeres. It was demonstrated that in leukemia cells induce the activation of α necrotic factor. Verbascoside has antimetastatic activities.[19] The beneficial properties, of which these metabolites are dispensers, seem to be strongly related to their peculiar power antioxidant and free radical scavenging, which allows to characterize these natural products as strong inhibitors of peroxides and processes to those of angiogenesis and tumorigenisis. Iridoids display an interesting spectrum of biological activity such as anti-inflammatory.[20] A few iridoid glycosides have demonstrated antioxidant properties. Picroside-I and kutkoside scavenged superoxide anions generated in a xanthine/xanthine oxidase system. These compounds are the main constituents of picroliv, the standard hepatoprotective preparation from *Picrorhiza kurroa*.[21] Their antioxidant capacity resulted from the prevention of the formation of free radicals. The iridoid glycoside geniposide was isolated together with the carotenoid crocin as a main antioxidant component of gardenia fruit.[22]

3. Experimental

3.1. General experiment procedures

NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian 300 spectrometer Fourier transform NMR in $CDCl_3$ or CD_3OD at 25 °C. The spectrum width was 2300 Hz. The initial matrix of 2 k × 2 k data points was zero-filled to give a final matrix of 4 k × 4 k points. The TOCSY experiments were performed in the phase-sensitive mode with a mixing time of 90 ms. The spectral width was 3000 Hz. The initial matrix of 512 × 512 data points was zero-filled to give a final matrix of 1 k × 1 k points. The NOESY experiments were performed in the phase-sensitive mode. The mixing time was 500 ms and the spectral width was 3000 Hz. The initial matrix of 512 × 512 data points was zero-filled to give a final matrix of 1 k × 1 k points. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimized for $^{1}J_{HC}$ = 140 Hz, a gradient heteronuclear multiple bond coherence (HMBC), optimized for $^{n}J_{HC}$ = 8 Hz. The HSQC experiment was performed in the phase sensitive mode with field gradient. The spectral width was 22,000 Hz in f_1 (^{13}C) and 3200 Hz in f_2 (^{1}H) and the matrix of 1 k × 1 k data points was zero-filled to give a final matrix of 2 k × 2 k points. The HMBC experiment was performed in the absolute value mode with field gradient. The spectral width was 20,000 Hz in f_1 (^{13}C) and 1100 Hz in f_2 (^{1}H) and the matrix of 1 k × 1 k data points was zero-filled to give a final matrix of 4 k × 4 k points. UV spectra were performed on UV-1700 Shimadzu spectrophotometer in MeOH solution. Optical rotations were measured on a Perkin–Elmer 141 (Perkin–Elmer Co., Norwalk, CT) in MeOH solution. The preparative HPLC apparatus consisted of Knauer Smartline 31/40 module equipped with Knauer Smartline 1000 pump, UV Knauer Smartline 2500 detector and RI Knauer Smartline 2300 detector and PC CromGate® *software*. Preparative HPLC were performed using Luna RP-8 (10 μm, 250 × 10.0 mm i.d., Phenomenex) and Luna RP-18 (10 μm, 250 × 10.0 mm i.d., Phenomenex). Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-8 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1.0 mm film thickness. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh), Merck Kieselgel 60 (40–63 μm) and Amberlite XAD-4 (Fluka).

4. Plant material

Plants of *T. chamaedrys* (Labiatae) were collected in May 2008, in the vegetative state, in the Natural Reserve of Castel Volturno, (Caserta, Italy), and identified by Dr. Assunta Esposito of the Second University of Naples. A voucher specimen (CE 0037) has been deposited in the Herbarium of the Dipartimento di Scienze della Vita of Second University of Naples.

5. Extraction and isolation

Leaves and roots of *T. chamaedrys* were dried in a ventilated thermostat at 45 °C for 5 days obtainaing a leaf dried material (761.3 g) and root one (19.9 g). The leaf dried material was infused first in hexane for 5 days, then in ethyl acetate for 5 days and finally in MeOH for 5 days in a refrigerated chamber at 4 °C, in the darkness. After removal of the solvents, we obtained crude hexane (6.2 g), EtOAc (13.1 g) and MeOH (107.9 g) extracts, which were stored at −80 °C until their purification. Analogously we obtained three root crude extracts: hexane (0.5 g), EtOAc (1.6 g) and MeOH (20.0 g). Both the methanol extracts were fractionated by liquid–liquid extraction. The obtained aqueous fraction was chromatographed on Amberlite XAD-4 eluting first with H_2O and then with MeOH.

The leaf organic fraction (27.0 g) from Amberlite XAD-4 was chromatographed by flash chromatography on SiO_2 eluting with the lower phase of a biphasic solution $CHCl_3$/MeOH/H_2O (13:7:2) to obtain two fractions A and B. Fraction A was chromatographed by RP-8 CC. One of the obtained fractions was purified by RP-8 HPLC using as eluent MeOH/H_2O (1:4) furnishing compounds **5** (41.2 mg) and **6** (17.2 mg). Fraction B was chromatographed by RP-18 CC eluting with MeOH/H_2O (1:4) to have pure metabolite **7** (8.3 mg).

The root organic fraction (7.0 g) from Amberlite XAD-4 was chromatographed by flash chromatography on SiO_2 eluting with the lower phase of $CHCl_3$/MeOH/H_2O biphasic solutions to obtain three fractions C–E. Fraction C, eluting with $CHCl_3$/MeOH/H_2O (13:9:5), was chromatographed by RP-18 HPLC using as mobile phase MeOH/MeCN/H_2O (5:1:4) to have pure compounds **1** (3.5 mg), **2** (14.2 mg), **3** (2.4 mg) and **4** (4.4 mg). Fraction D, eluting with $CHCl_3$/MeOH/H_2O (13:9:4), was chromatographed by RP-18 HPLC using as eluent MeOH/MeCN/H_2O (3:1:6) to obtain pure compounds **9** (12.3 mg), **10** (1.7 mg) and **12** (3.6 mg). Fraction E, eluting with $CHCl_3$/MeOH/H_2O (13:7:2), was chromatographed by RP-18 HPLC [MeOH/MeCN/H_2O (7:2:11)] to have pure metabolites **8** (9.3 mg) and **11** (4.3 mg).

5.1. 6′-(3,4-Dimethoxycinnamoyl)ayugol (1)

$[\alpha]_D^{25}$ −27.3 (*c* 0.15, MeOH). UV (MeOH) λ_{max} (log ε) 322.5 (4.26) nm. ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 75 MHz) data, see Table 2. Anal. Calcd for $C_{26}H_{34}O_{12}$: C, 57.99; H, 6.36. Found: C, 58.00; H, 6.34.

5.2. 6′(3,4-Dimethoxycinnamoyl)harpagide (2)

$[\alpha]_D^{25}$ −22.6 (*c* 0.71, MeOH). UV (MeOH) λ_{max} (log ε) 323.5 (4.34) nm. ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 75 MHz) data, see Table 2. Anal. Calcd for $C_{26}H_{34}O_{13}$: C, 57.99; H, 6.36. Found: C, 58.00; H, 6.34.

5.3. 2′-*O*-β-D-Glucopyranosil-6′(*p*-methoxycinnamoyl)harpagide (3)

$[\alpha]_D^{25}$ −27.1 (*c* 0.21, MeOH). UV (MeOH) λ_{max} (log ε) 226.6, 311.0 (3.63, 3.79) nm. ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD,

75 MHz) data, see Table 2. Anal. Calcd for $C_{31}H_{42}O_{17}$: C, 57.99; H, 6.36. Found: C, 58.00; H, 6.34.

5.4. 1-(3,4-Dihydroxyphenylethyl)-*O*-α-L-lyxopyranosil-(1→2)-α-L-rhamnopyranosil-(1→3)-6-*O*-transferuloyl-β-D-glucopyranoside (12)

$[\alpha]_D^{25}$ −15.5 (*c* 0.18, MeOH). UV (MeOH) λ_{max} (log ε) 306.5 (3.72) nm. ^{1}H NMR (CD_3OD, 300 MHz) data: 7.62 (d, 1H, *J* = 15.9 Hz), 7.15 (d, 1H, *J* = 1.8 Hz), 7.02 (dd, 1H, *J* = 8.4 and 2.1 Hz), 6.80 (d, 1H, *J* = 8.4 Hz), 6.66 (d, 1H, *J* = 1.8 Hz), 6.62 (d, 1H, *J* = 8.1 Hz), 6.52 (dd, 1H, *J* = 8.0 and 1.8 Hz), 6.38 (d, 1H, *J* = 15.9 Hz), 5.40 (d, 1H, *J* = 1.8 Hz), 4.92 (t, 1H, *J* = 9.2 Hz), 4.90 (d, 1H, *J* = 3.6 Hz), 4.51 (ov, 1H); 4.35 (dd, 1H, *J* = 11.5 and 2.5 Hz), 4.32 (d, 1H, *J* = 8.1 Hz), 3.87 (dd, 1H, *J* = 3.1 and 1.8 Hz), 3.97 (m, 1H), 3.88 (ov, 1H), 3.86 (s, 3H), 3.75 (m, 2H), 3.72 (dd, 1H, *J* = 8.0 and 3.6 Hz), 3.72 (ov, 1H), 3.66 (ov, 1H), 3.52 (m, 2H), 3.51 (s, 1H), 3.30 (ov, 1H), 3.29 (dd, 1H, *J* = 9.8 and 9.7 Hz), 2.78 (t, 2H, 7.5 Hz), 1.24 (d, 1H, *J* = 6.6 Hz); ^{13}C NMR (CD_3OD, 75 MHz) data: 169.1, 149.3, 147.1, 147.0, 144.7, 144.4, 131.3, 127.6, 124.3, 121.3, 121.2, 117.0, 116.4, 116.3, 115.2, 104.4, 104.3, 101.4, 83.8, 80.4, 75.4, 75.1, 74.3, 72.6, 72.2, 72.0, 71.4, 70.3, 70.0, 68.9, 64.6, 56.4, 36.7, 17.9. Anal. Calcd for $C_{35}H_{46}O_{19}$: C, 54.54; H, 6.02. Found: C, 54.51; H, 5.99.

6. Antioxidant capability determination

6.1. DPPH radical scavenging capacity[7]

Evaluating the scavenging activity of the extracts, rates of each extract (4.2, 8.3, 16.7, 25, 33.3, 83.3 μg; 250 μL) or pure isolated metabolites (5, 10, 20 μM; 150 μL) were added to methanolic DPPH· solution (9.4×10^{-5} M, Fluka Chemie) at room temperature to have 1.5 mL as final volume. The absorbance at 515 nm was measured at 30 min against a blank using a UV-1700 Shimadzu spectrophotometer. The analyses were carried out in triplicate. The results are expressed in terms of the percentage reduction of the initial DPPH radical adsorption by the test samples. The DPPH radical scavenging activity has been compared with those exercised by Trolox® used as standard. The extracts IC_{50} value was also determined.

6.2. Determination of TBARS[7]

Olive oil (10.0 μg) was emulsified with 30.0 mg of Tween-40 (Fluka Chemie) initially dissolved in 1.5 ml of 0.2 M Tris–HCl buffer, pH 7.4. The emulsion was irradiated with UV light at 254 nm for 60 min and then stirred for 24 h at room temperature. Then, test compound (5, 10, 20 μM; 150 μL) was added to the reaction mixture and exposed to UV light for 60 min again. TBA reagent (2.0 ml), prepared dissolving 375 mg of thiobarbituric acid, 30 mg of tannic acid and 15 mg of trichloracetic acid in of hydrogen chloride aqueous solution (0.1 L, 0.2 M), was added. Test tubes were placed into a boiling water bath for 90 min and then centrifuged using a Beckman GS-15R centrifuge for 3 min at 1500 rpm. The supernatant was measured at 532 nm by Shimadzu UV-1700 spectrophotometer. Inhibition of test lipid peroxidation was measured as a percentage against a blank containing no test compounds. The analyses were carried out in triplicate. IC_{50} value and corresponding TEAC (Trolox® equivalent antioxidant capacity) value were also estimated.

6.3. Determination of effects on oxidation of deoxyribose

The determination of effects on oxidation of deoxyribose was carried out as described by Halliwell et al.[23] with some modifications. The reaction mixture containing EDTA (1.0 mM), $FeCl_3$ (1.0 mM) and H_2O_2 (1.0 mM) was incubated at 37 °C for 1 h. Then 2-deoxyribose (28 mM) and ascorbic acid (1.0 mM) were added. Pure test compound (5, 10, 20 μM; 150 μL) was added to 1350 μL of mixture. The performed system was incubated at 37 °C for 24 h. The extent of 2-deoxyribose degradation was tested by using the TBA method. TBA (1.0 mL; 0.5%, w/v) and TCA (1 mL; 2.5%, w/v) were added to the mixture, which was heated in a water bath at 90 °C for 3 h. The absorbance of the mixture was read spectrophotometrically at 532 nm. The analyses were carried out in triplicate. The detected activities are reported as antioxidant capacity percentage vs blank. IC_{50} value and corresponding TEAC (Trolox® equivalent antioxidant capacity) value were also estimated.

6.4. Determination of effects on protein oxidation

The effects of metabolites **2**–**12** on protein oxidation were determined according to the method of Levine et al.[24] with some modifications. The reaction mixture (1.5 mL) in phosphate buffer (20 mM, pH 7.4) containing pure metabolite (5, 10, 20 μM; 150 μL), bovine serum albumin (10 mg/mL), $FeCl_3$ (400 μM), H_2O_2 (3 mM) and ascorbic acid (400 μM) was incubated at 37 °C for 1 h. Dinitrophenyl hydrazine (DNPH, 1 mL, 20 mM in HCl 2 N) and 1 mL of trichloroacetic acid (TCA, 20% w/v) were added to the reaction mixture. The mixture was centrifuged at 1500 rpm for 10 min. The protein was washed first three times with 2 mL of EtOH/EtOAc (1:1) solution and then was dissolved in 2 mL of 6 M guanidine–HCl (pH 6.5). The absorbance of the sample was read at 370 nm. The analyses were carried out in triplicate. The detected activities are reported as antioxidant capacity percentage vs blank. IC_{50} value and corresponding TEAC (Trolox® equivalent antioxidant capacity) value were also estimated.

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