American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

American Journal of Food Technology 2 (7): 618-629, 2007
ISSN 1557-4571

Synthesis and Antioxidant Activity of Modified (+)-Catechin Derivatives. Structure-Activity Relationship

[1,2]Nour-Eddine Es-Safi, [1]Josiane Beauhaire,
[3]Christine Le Guernevé and [1]Paul-Henri Ducrot
[1]Unité de Phytopharmacie et Médiateurs Chimiques, INRA,
Route de Saint-Cyr, 78026 Versailles Cedex, France
[2]Laboratoire de Chimie Organique et d'Etudes Physico-Chimiques, Pôle de Compétences Pharmacochimie, Ecole Normale Supérieure, B.P 5118 Takaddoum Rabat, Morocco
[3]UMR Sciences Pour l'œnologie, INRA, 2 place Viala 34060 Montpellier Cedex, France

Abstract: In a screening program for free radical scavengers, new antioxidant compounds have been obtained by C-8 substitution of various groups on (+)-catechin A ring. Their preparation, purification, identification by MS, ^{1}H and ^{13}C NMR analysis as well as their antiradical/antioxidant properties are reported. In order to explore the substituent effect on the observed activity, the obtained results were compared to that of (+)-catechin. Some of the new compounds were more efficient than the underivatised (+)-catechin as scavengers of 1,1-diphenyl-2-picrylhydrazyl (DPPH°) free radical.

Key words: Polyphenols, catechin, flavan-3-ols, modified flavan-3-ols, synthesis, characterization, antioxidant activity, free radical scavenging, DPPH°

INTRODUCTION

Polyphenols are ubiquitous plant compounds increasingly appreciated as chemopreventive agents against several diseases such as cancer, cardiovascular and neurodegenerative diseases (Katiyar and Mokhtar, 1997; Fremont, 2000; Fuhram and Aviram, 2001; Yang *et al*., 2001; Rohdewald, 2002; Aruoma *et al*., 2003; Dajas *et al*., 2003; Murphy *et al*., 2003; Scalbert *et al*., 2005). Among all these beneficial effects, the primary activity of plant polyphenols is believed to reside in their free radical scavenging capacity (Rice-Evans *et al*., 1996; Yokozawa *et al*., 1998; Kroon and Williamson, 2005), which is the most important reported biological property of flavonoids, by scavenging oxygen radicals and inhibiting peroxidation (Hanasaki *et al*., 1994). Antioxidants are compounds that delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. In the food industry, they have long been used as additives to protect food products from oxidation-related quality deterioration such as rancidity and off-flavors (Sanchez-Moreno *et al*., 1998; Lee *et al*., 2004).

It has been reported that antioxidant activities primarily originate from compounds with phenolic structures (Rice-Evans *et al*., 1997; Ismail *et al*., 2004; Halliwell, 2006), indicating a significant positive relationship between total phenolic content and antioxidant activity (Velioglu *et al*., 1998). Among these phenolics, catechins have been the subject of several studies due to their contribution to the antioxidant properties of tea beverages (Serafini *et al*., 1994), which have demonstrated protective effects against cardio-vascular and liver diseases (Imai and Nakachi, 1995). Catechins were also found useful as food preservatives which prevent the formation of toxic oxygen species and peroxides in food (Nakayama *et al*., 1994).

Corresponding Author: Nour-Eddine Es-Safi, Laboratoire de Chimie Organique et d'Etudes Physico-Chimiques, Pôle de Compétences Pharmacochimie. Ecole Normale Supérieure, B.P 5118 Takaddoum Rabat, Morocco

The antioxidant activity of polyphenols is considered to be due to their properties as free radical terminators. This activity depends mainly on different structural features such as O-H bound dissociation energy, resonance delocalisation of the phenol radical and steric hindrance derived from hydrogen substitution in the aromatic ring (Shahidi and Naczk, 1995). The effect of polyphenols antioxidants on DPPH° free radical scavenging is thought to be primary due to their hydrogen donating ability owing to the presence of hydroxyl groups (Baumann *et al*., 1979).

Interestingly, the non-phenolic part of the molecule appears also to influence the activity of the conjugates, particularly their capacity to penetrate biological membranes, as well as the layers of the skin (Alonso *et al*., 2004). Thus flavan-3-ol conjugates with thiols have been showed to exhibit a higher antiradical capacity than their counterparts in the DPPH° assay (Tanaka *et al*., 1998; Torres and Bobet, 2001; Torres *et al*., 2002a, b, 2005; Lozano *et al*., 2005) suggesting the favourable role played by the non-phenolic part of the molecules in the antioxidant activity. This prompted us to investigate whether the introduction of a substituent in the 8 position of the (+)-catechin A ring influences the protective efficacy of flavanols against free radicals.

We report in this study the preparation, isolation structure elucidation and the antioxidative activity of conjugates of (+)-catechin which are significantly more effective than the underivatised flavan-3-ol suggesting that the non phenolic part of the molecule also plays a significant role in antioxidative protection. To our knowledge these compounds have not been previously investigated for their free radical scavenging activity.

MATERIALS AND METHODS

Generals

All reactions were performed under argon and monitored by TLC and analytical HPLC. Otherwise indicated, the ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ with a Varian Gemini-300 spectrometer at 300 and 75 MHZ, respectively (proton decoupling mode for carbon). ^{1}H NMR spectra were referenced to the signal at $\delta = 7.27$ ppm of residual $CHCl_3$. ^{13}C NMR spectra were referenced to signals of $CDCl_3$ ($\delta = 77.0$ ppm). Resonances of the benzyl group are not mentioned. FT-IR spectra were recorded with a Nicolet Avatar 320 FT-IR spectrophotometer. UV-visible Spectra were recorded with a Kontron Uvikon 930 spectrophotometer fitted with a quartz cell.

Analytical TLC was performed on Merck silica gel 60 F254 plates. Column chromatography was performed using a mixture of ethyl acetate/cyclohexane as eluent was performed on silica gel 60 Å 70-200 μm (SDS, 13124 PEYPIN, France). Analytical HPLC analysis was performed on a Varian apparatus including a 9012 solvent delivery system, a 9100 autosampler and a 9065 polychrom diode array detector. Analysis were performed on a C18 column eluting with a mixture of solvents A: acetonitrile and B: water with 0.5% orthophospharetic acid eluting from 15 to 100% A in 18 min followed by a washing and a reequilibrating of the column. LC/MS analysis were performed with a chromatographic system (Alliance) consisted of a Waters 2695 separations module equipped with an autosampler and a Waters 2487 dual lambda absorbance detector (Waters, Milford, MA, USA). The column was a 150×2.1 mm Interchrom UP5ODB#15E (Uptisphere 5 μm ODB) with a 10×2.1 mm precolumn from Interchim (Montluçon, France). Chromatography was ran in isocratic mode using a 60/40 mixture of acetonitrile (RS-Plus quality for HPLC from Carlo Erba) and water with 0.2% acetic acid. The flow rate was 0.2 mL min^{-1}, the analyses were performed with the column and the samples kept at ambient temperature and 5.0-10 μL was injected for each analysis. The effluent from the UV detector was introduced without any split into the mass spectrometer. The HPLC system was coupled on line to a Quattro LC MS/MS triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a pneumatically assisted Electrospray Source Ionisation (ESI). Data acquisition and processing were performed using a MassLynx NT 3.5 data system. The electrospray source

parameters were fixed as follow: electrospray capillary voltage 3.25 kV in positive mode and 3 kV in negative mode, source block temperature 120°C, desolvatation gas temperature 400°C. Nitrogen was used as drying gas and nebulising gas at flow rates of approximately 50 and 450 L h^{-1}.

Reduction of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH°) Radical

The free radical scavenging activity was evaluated by the DPPH method (Brand-Williams *et al*., 1995; Torres *et al*., 2002a). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with 4.8 mg DPPH° in 200 mL of MeOH and the mixture incubated for 1 h at room temperature. The initial concentration of DPPH°, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH° at different concentrations. The studied compounds were tested with MeOH as negative control and BHT and quercetin as positive control and absorbance at 517 nm was determined. The absorbance (A) of the control and samples was measured and the DPPH scavenging activity in percentage was determined as follow:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}]\times 100$$

The data are presented as mean of triplicate and the concentration required for a 50% reduction (IC_{50}) of DPPH° radical was determined graphically.

RESULTS AND DISCUSSION

Synthesis of Modified (+)-Catechin Derivatives

We started investigations on the synthesis of new modified monomeric units derived from catechin with the objectives of exploring the impact of the A ring substitution on their biological properties. For this study, the six 8-substituted derivatives of flavan-3-ols 3-8 in addition to taxifolin 9 were synthesized and their antioxidative activity investigated. The modified flavan-3-ols derivatives described in this work are depicted in Fig. 1 and their synthesis pathways are depicted in Fig. 2. As can be noticed, the studied compounds were synthesized in their benzylated forms 11, 12, 14-16 and 19. Their free forms were obviously obtained through deprotection in a MeOH/THF medium, under H_2 and in presence of Pd as catalyst.

Compound 11 was prepared by action of trifluoacetic anhydride on tetrabenzylated catechin 10 following a Friedel-Craft's reaction on the 8 nucleophile position as previously described (Beauhaire *et al*., 2005; Es-Safi *et al*., 2006). Further reduction by $NaBH_4$ afforded compound 12. Compound 14 was prepared starting from the pentabenzylated catechin 13. After formylation through the classical Vilsmeier reaction, the obtained compound 14 was reduced by $LiAlH_4$ giving the hydroxymethyl derivative 15. Further reduction of the latter gave the target product 16 with a good yield. The bromided adduct 17 was obtained from 13 by action of NBS. Gram-scale of taxifolin 19 was prepared from (+)-catechin through reactions involving oxidation processes as previously described (Es-Safi and Ducrot, 2006).

The structures of these modified catechin derivatives were determined through UV, MS and NMR spectroscopy. Structure elucidation of compound 16 will be detailed in this paper as example. The ESI-MS spectrum recorded in the positive ion mode showed signals located at m/z: 755, 772 and 777 corresponding to $[M+H]^+$, $[M+NH_4]^+$ and [M+Na] ions respectively and indicating a molecular weight of 754 amu in agreement with the structure of compound 16. The usual flavan-3-ols characteristic RDA fragmentation was also observed at m/z: 423, $[M+H-332]^+$ ion and corresponding to the protonated A moiety (Fig. 3).

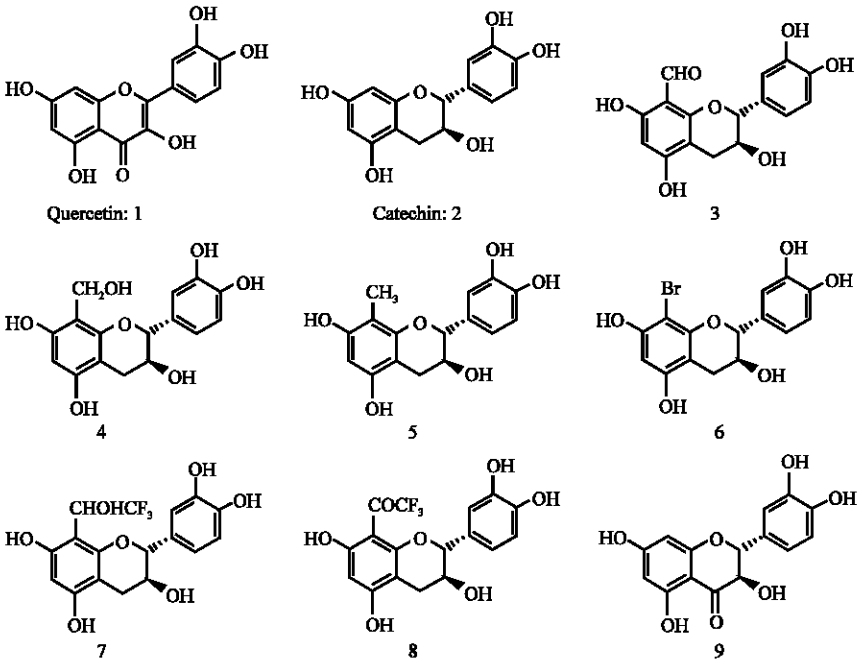

Fig. 1: Structures of the studied polyphenols

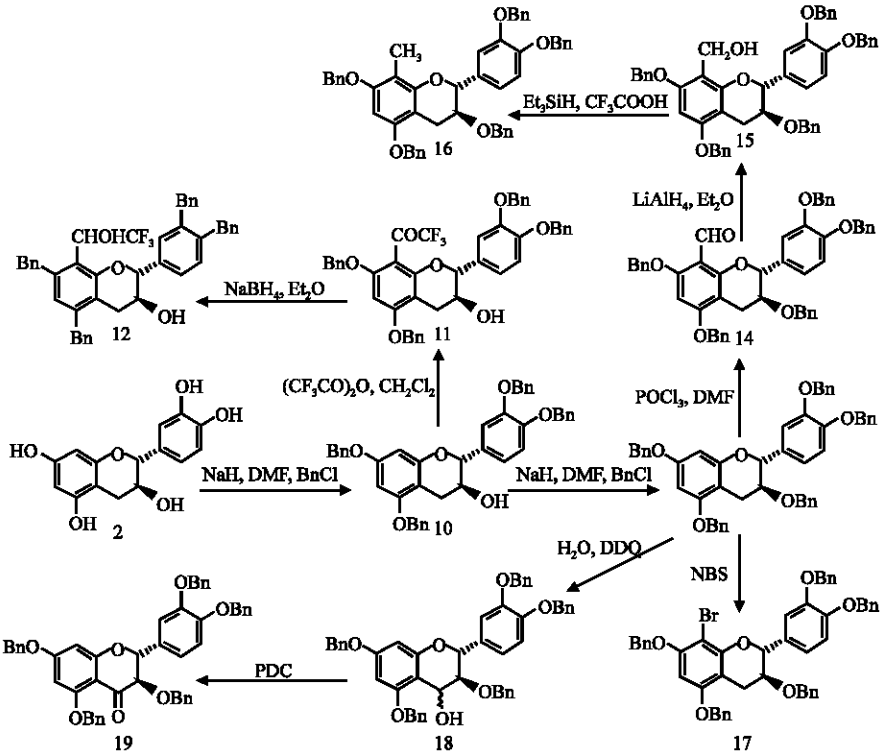

Fig. 2: Synthesis pathways of the studied modified flavan-3-ols

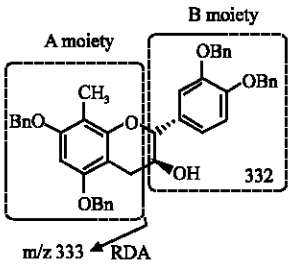

Fig. 3: Main MS/MS fragmentations observed in compound 16

The remaining outstanding question that needed to be resolved was related to the position of the methyl group on the flavanol A ring. This constitutes the most encountered problem in flavanols structural characterization. Since the two positions 6 and 8 are almost magnetically equivalent, they could not be distinguished on the basis of their chemical shift. In our case, assuming that the substitution occurs at the more nucleophilic positions of the flavanol skeleton, i.e., 6 or the 8 as confirmed through ES-MS spectrometry, determination of the residual proton (H6 or H8) could not be achieved based only on its chemical shift but would rather requires the use of 2D NMR analysis.

The position of the CH_3 group on the aromatic A ring was thus elucidated by long range distance carbon-proton correlations established by 2D NMR HMBC experiments through the following reasoning. The usual pyran ring protons H4 [(2.71 ppm, dd, J = 16.69 and 5.59 Hz) and 3.03 ppm (dd, J = 16.69 and 8.73 Hz)], H3 (6.60 ppm, m) and H2 (7.90 ppm, d, J = 7.99 Hz) were easily assigned by ^{1}H NMR analysis. The three B ring protons were observed between 6.93 and 7.00 ppm. For the aromatic A ring, only one proton signal appearing as a singlet at 6.21 ppm was present indicating a monosubstitution. The presence of the CH_3 group was confirmed through ^{1}H NMR analysis showing a singlet at 2.06 ppm. The protonated carbon chemical shifts were assigned through NMR HSQC analysis.

The definitive structure elucidation of compound 16 was achieved by HMBC experiment which allowed assignment of all hydrogen and carbon atoms. In addition to their correlations with C2 (79.84 ppm) and C3 (74.97 ppm), H4 protons (2.77 and 3.03 ppm) correlated with 3 carbons located at 102.74, 153.13 and 154.75 ppm. Carbons C4a, C8a and C5 are in a favorable position to give such correlations (Fig. 4). The signal observed at 102.74 ppm was attributed to C4a due to its chemical shift position compared to C8a and C5 which are linked to an oxygen atom. The carbon signal located at 153.13 ppm also gave a correlation with H2, which pointed to the C8a carbon and thus the remaining signal observed at 154.75 ppm was attributed to C5. The C8a signal thus attributed did not show any correlation with the residual A ring aromatic protons which is thus H6. This was also confirmed by the presence of a correlation between C5 and the residual aromatic proton and between the methyl protons and the C8a (Fig. 4). The position of the methyl group on the A ring 8 position was thus demonstrated.

Antioxidative Activity of the Synthesized Compounds

Free radicals are known to be a major factor in biological damages and DPPH° has been used to evaluate the free radical-scavenging activity of natural antioxidants (Yokozawa *et al*., 1998). DPPH°, which is a molecule containing a stable free radical with a purple color, changes into a stable compound with a yellow color by reacting with an antioxidant which can donate an electron to DPPH°. In such case, the purple color typical of the free DPPH° radical decays, a change which can be followed either spectrophotometrically (517 nm) or by detecting changes in concentration of starting materials and/or end reaction products, using HPLC analysis (Belinky *et al*., 1998).

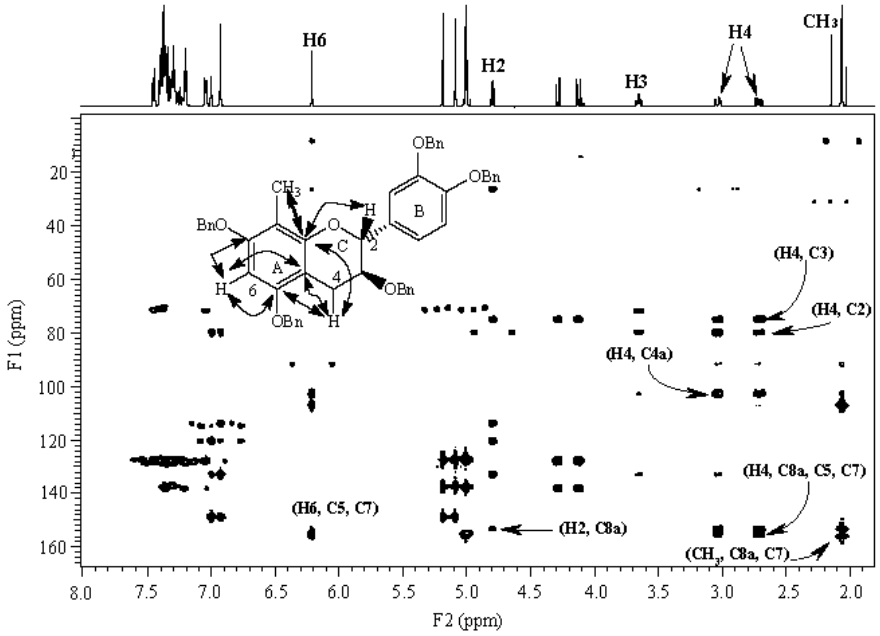

Fig. 4: HMBC spectrum and long range correlations observed in compound 16

The fact that the extent of the reaction depends on the hydrogen donating ability of the antioxidant (Bondet *et al*., 1997) make of it an indication of the capacity of the tested products to scavenge free radicals. This simple test can provide information on the ability of a compound to donate an electron, the number of electrons a given molecule can donate and on the mechanism of antioxidant action. In cases where the structure of the electron donor is not known (e.g., a plant extract), this method can afford data on the reduction potential of unknown materials. The DPPH° test is a very convenient method for screening small antioxidant molecules because the reaction can be observed visually using common TLC and also its intensity can be analysed by simple spectrophotometric assays (Sanchez-Moreno *et al*., 1998). The DPPH° radical is scavenged by antioxidants through the donation of hydrogen to form the stable reduced DPPH molecule. The antioxidant radicals formed are stabilized through the formation of non-radical products. This method has been also a useful and widely used to evaluate the free radical-scavenging effectiveness of various antioxidant substances in food systems (Cotelle *et al*., 1996).

In order to investigate the antioxidant activities of the synthesized modified (+)-catechin derivatives the DPPH° method was used and the results obtained are depicted in Fig. 5. It shows the DPPH° free radical scavenging activity of each compound at a concentration of 10 μM where a general decrease in the absorbance at 517 nm was noticed. This indicated a DPPH° free radical-scavenging activities of the tested compounds. It can be also noticed that most of the synthesized compounds showed obvious scavenging activity on DPPH° radicals. All the modified flavan-3-ols displayed stronger activities than that of BHT and most of them showed better antioxidative activity than (+)-catechin. Among the tested modified flavan-3-ols, compounds 7 ($CHOHCF_3$), 8 ($COCF_3$) and 9 (taxifolin) displayed the strongest activity with an absorbance decrease of about 43, 44 and 45%, respectively. And the order of the activities of flavan-3-ol derivatives were showed to be as follow

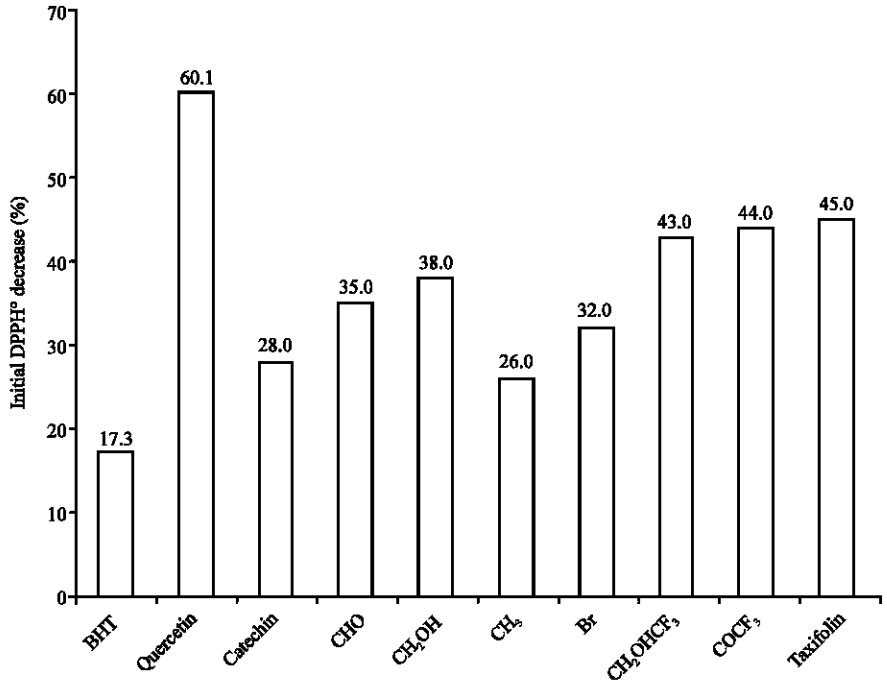

Fig. 5: Free radical scavenging activity of the studied compounds. The results represent the decrease (%) of the initial DPPH° absorption at 517 nm

9 (taxifolin) > 8 ($COCF_3$) >7 ($CHOHCF_3$) >4 (CH_2OH) >3 (CHO) >6 (Br) >2 (catechin) >5 (CH_3), that is, the modified flavan-3-ols with oxygenated and/or hydroxylated substituents showed much higher antioxidant activities than that of (+)-catechin.

The free radical scavenging activity is usually expressed as percentage of DPPH° inhibition but also by the antioxidant concentration required for a 50% DPPH° reduction (IC_{50}). A dose-response curve was thus obtained for every product where all the tested compounds showed dose-dependant increase in activity and a spectrophotometric analysis was used in order to determine the inhibition concentration (IC_{50}) of the studied samples, which is a widely used parameter for determining radical scavenging capacity of pure samples. IC_{50} is the amount of antioxidant necessary to determine the initial concentration of DPPH° radical by 50 %. IC_{50} value is considered to be a good measure of the antioxidant efficiency of pure compounds and extracts.

The obtained results are summarized in Fig. 6 which showed that the new flavan-3-ol derivatives are potent free radical scavenging agents in the DPPH° free radical assay. From IC_{50} the ED_{50} is determined. ED_{50} represents the number of products (µmole) able to consume half the amount of free radical divided by µmole if initial DPPH°. The inverse of ED_{50} is a measure of the antiradical power (ARP). By multiplying the ED_{50} by two, the stoichiometric value (theoretical concentration of antioxidant to reduce 100 % of the DPPH°) is obtained. The inverse of this value represents the moles of DPPH° reduced by one mole of antioxidant and gives an estimate of the number of hydrogen atoms involved in the process. Table 1 summarizes the parameters obtained. It can be noticed that some of the tested compounds were more efficient than (+)-catechin 2 and BHT, being the order of antiradical power 9 (taxifolin) > 8 ($COCF_3$) >7 ($CHOHCF_3$) >4 (CH_2OH) >3 (CHO) >6 (Br) >2 (catechin) >5 (CH_3). The stoichiometric value obtained for (+)-catechin was 0.36 corresponding to the reduction of ca. three DPPH°. Most of the modified tested flavan-3-ols were able to reduce roughly one more

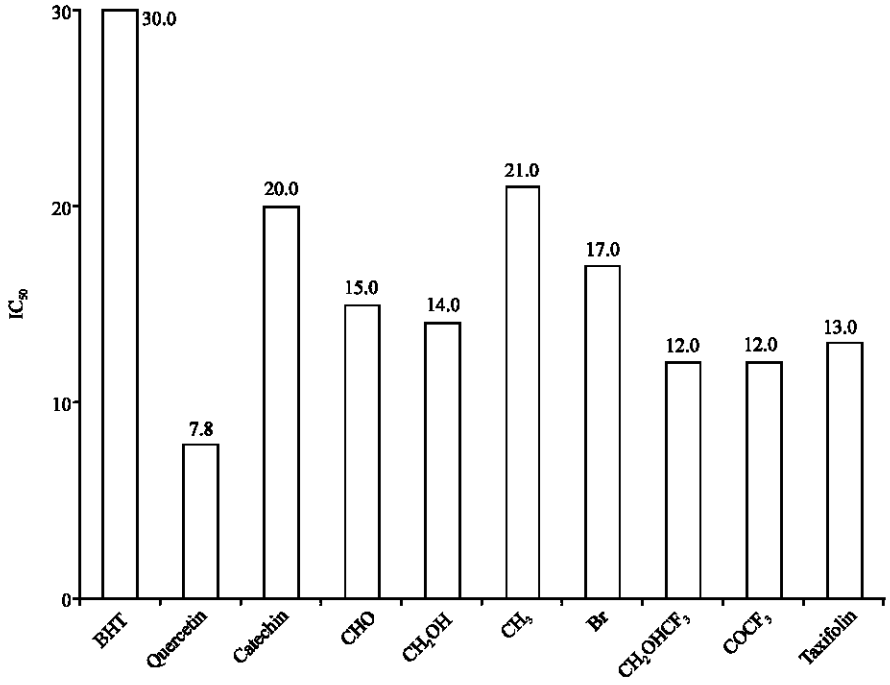

Fig. 6: Free radical scavenging activity of the studied compounds. The results represent the concentration IC_{50} needed to decrease by 50% the initial DPPH° absorption at 517 nm

molecule of DPPH° than the underivatised (+)-catechin. Compound 9 is clearly the most efficient of the new tested molecules. Among all the tested compounds, only compound 5 with a methyl group as substituent was less active than (+)-catechin. Quercetin with five hydroxyl groups, donated an average of 4.8 electrons/molecule. Results shown in Table 1 demonstrate that flavonoids which react with DPPH° and donate an electron are those with an oxygenated substituent.

Structure-Activity Relationship

Many attempts have been reported in the literature to explain the structure-activity relationship of some natural antioxidant compounds. It has been reported that the antioxidant activity of phenolic compounds may result from the neutralization of free radicals initiating oxidation processes, or from the termination of radical chain reactions, due to their hydrogen donating ability (Baumann *et al*., 1979). It is also known that the antioxidant activity of polyphenolic compounds is closely associated with their structures, such as substitutions on the aromatic ring and side chain structure. Their accessibility to the radical centre of DPPH° could also influence the order of the antioxidant power. Free radical-scavenging activity of polyphenolic compounds is believed to be influenced by the number and position of phenolic hydrogen in their molecules (Rice-Evans *et al*., 1996). It is also proposed that the higher antioxidant activity is related to the greater number of hydroxyl groups on the flavonoid nucleus (Cao *et al*., 1997).

In overall agreement with structure-activity relationship studies of the free radical scavenging capacity of flavonoids (Bors *et al*., 1990; Rice-Evans *et al*., 1996), several structural features were shown to be important for the protective effect of flavonoids against glutamate-mediated programmed cell death (Ishige *et al*., 2001) including the presence of a hydroxyl group on C-3 and a 2-3 double bond

Table 1: Antiradical power and stoichiometry from the DPPH assay

Compound	ED_{50}	ARP	Stoichiometric value	H atoms per molecule
BHT	0.33	3.00	0.67	1.50
1 : Quercetin	0.10	9.60	0.21	4.80
2 : (+)-Catechin	0.18	5.60	0.36	2.80
3: (CHO)	0.14	7.00	0.29	3.50
4: (CH_2OH)	0.14	7.20	0.28	3.60
5: (CH_3)	0.19	5.20	0.38	2.60
6: (Br)	0.15	6.60	0.30	3.30
7: ($CHOHCF_3$)	0.14	7.40	0.27	3.70
8: ($COCF_3$)	0.13	7.60	0.26	3.80
9: Taxifolin	0.13	8.00	0.25	4.00

in conjugation with a C-4 ketone function. The formation of hydrogen bonds between the ketonic oxygen and the hydroxyl at C-3 and C-5 may have some influence on the scavenging power as well (Bors *et al*., 1990).

Catechins do not contain either the unsaturation or the ketone function on C-4 and this is the reason why compounds such as (+)-catechin 2 are less potent scavengers than flavonols such as quercetin. Some catechins such as galloylated or oligomeric derivatives compensate for this by the presence of more hydroxyl groups as well as the ester function at C-3 (Rice-Evans *et al*., 1996; Bors *et al*., 2000, 2001).

While the antioxidative activity of (+)-catechin and its oligomeric forms (procyanidins) was investigated, little is known about the antioxidant activity of 8-modified flavan-3-ols. As shown in Table 1, only one compounds 5 exhibited antioxidant activity lower than (+)-catechin, while the results obtained for all the tested compounds were higher than that of (+)-catechin and BHT. In the same conditions quercetin was the most potent antioxidative compound. The substituents on C-8 position of the aromatic A ring appears to have a clear influence of the antioxidant effect of (+)-catechin. The presence of a group on C-8, which allow for the formation of hydrogen bonds with the hydroxyl either C-7 may play a role in the ability of the (+)-catechin conjugates to scavenge free radicals in a manner similar to quercetin. Since the substituent moieties are attached to the side-chain hydroxyl group functionality of the flavanol skeleton, it is unlikely that they affect the aromatic hydroxyl group that is responsible for antioxidant activity.

Flavonoid hydrophobicity has also been proposed to play an important role the free radical scavenging activity. In view of the chemical structure of the compounds active, hydrophobicity may be most crucial. Instead, the 8-substitutents influence might also influence the polarity of the compounds becoming more or less hydrophobic and thus might give negative/positive influences on their antioxidant activity when assayed in aqueous conditions. This may explain why compound 5 with a methyl group presents a lower free radical scavenging activity than (+)-catechin.

CONCLUSION

In summary, the modified (+)-catechin derivatives obtained by substitution with different groups at the 8 position yielded compounds presenting relatively higher free radical scavenging activity. These compounds could thus protect human cells from oxidative stress-induced death. The pure new synthesized compounds proved to be potent free radical scavengers in the DPPH free radical assay. The antiradical efficacies of the studied compounds were compared with those of quercetin, BHT and (+)-catechin. The obtained results showed that the introduction of a substituent onto position 8 of (+)-catechin yielded compounds with improved antiradical efficacy in solution. The antiradical activity of (+)-catechin is exerted through the formation of semiquinone free radicals and quinines, triggered by the abstraction of one proton and one electron from some of the phenolic hydroxyl groups

(Guo *et al*., 1996; Kondo *et al*., 1999). The various substituents group of the new derivatives might modulate the reactivity of the molecules. Their oxidation might contribute to this antioxidant effect.

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