ESTABLISHMENT OF THE ANTIOXIDANT/ANTIRADICAL ACTIVITY OF THE INHIBITORS USING THE DPPH – RADICAL

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Abstract. This research paper presents the results of the investigation of antioxidant activities of various inhibitors, which are constituents of winery products: quercitin, rezveratrol, dihydroxyfumaric acid. Also, the antioxidant activity of tartaric and dihydroxyfumaric (DFH_4) acids derivatives has been determined: sodium dihydroxyfumarate, dimethylic ester of DFH_4 and dimethylic ester of tartaric acid. The enotannin extracts obtained from grape seeds have been evaluated: the non-oxidized enotannin extract *Eneox* and the oxidized one -*Enoxil.* For the determination of the antioxidant/antiradical activity the 2,2-diphenil-1-picrylhidrazil (DPPH) radical was used, which has the absorption maxima at 517 nm. The efficient concentration EC_{50}, the stoichiometric value of the antioxidant and the free radical, the antiradical power ($1/EC_{50}$) and the number of moles of DPPH-radical reduced by one mole of inhibitor have been calculated for all investigated inhibitors. It was found that catechin, quercitin and DFH_4 exhibit the highest inhibition rate.

Keywords: antioxidant activity, DPPH radical, inhibition rate, natural inhibitors from secondary winery materials

Overview

Numerous practical and epidemiological studies have confirmed that the micronutrients, thus the antioxidants existing in aliments, are able to inhibit the cancerigenesis through their influence on the molecular level, during the initiation, promotion and progression stages.

In recent times, the most studied compounds were the polyphenols, which are constituents of the plants.

The antioxidant activity of the polyphenols is controlled by the presence of the hydroxylic groups in the B ring in 3' and 4' positions and in a lesser degree, by the presence of the hydroxylic group from the C ring in 4' position.

The flavonols, especially catechin, quercitin, kaempherol and their glucosides are elements of the black and green teas [5] and red wine [5]. The diets rich in vegetables and fruits, especially in grapes, protect against heart diseases, various forms of cancer [7, 8], methemoglobinemy, and display anti-inflammatory, as well as antimutagenic effects [9]. These protective upshots were attributed, in a great measure, to the antioxidants that include flavonoids as well as carotenoids and vitamins C and B.

The majority of the polyphenolic constituents from products (flavonols – like quercitine and kaempherol, flavones – like luteolin, flavonones – like catechin, anthocyanidin, for example, cyanidin and malvidin and their glycosides) presents high efficiency, in comparison to the nutrient antioxidants: vitamins C, E, β-carotene, that are easily adsorbed in the intestines [10].

The grapes and the wine contain high concentrations of antioxidants. Based on the study of the overall antioxidant activity of the red wine, we concluded that 54,76% is determined by the contribution of catechin and epicatechin, that form approximately 63,54% of the phenolic constituents (191 and 82 mg/l, correspondingly) [10].

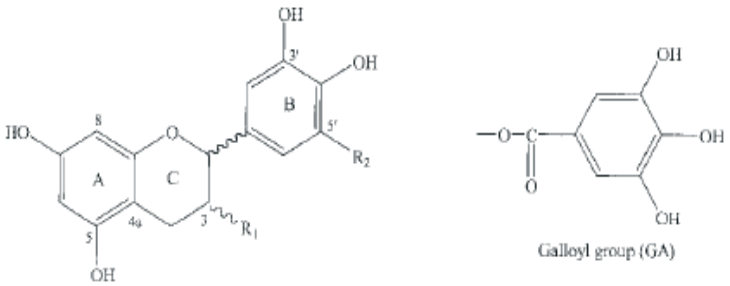


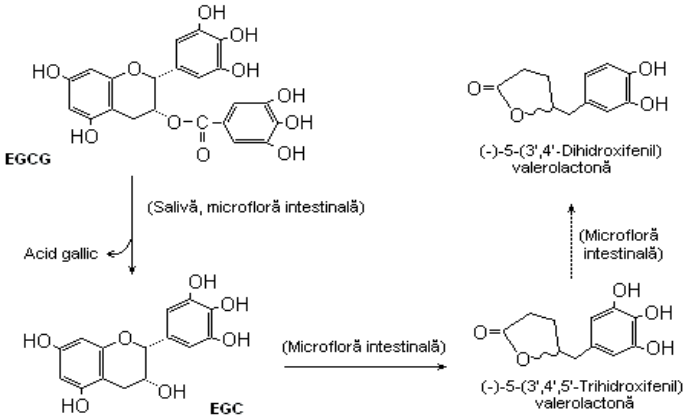
Fig.1. Chemical structure of the catechins present in tea and wine [5]. Catechin-(R_1 - OH, R_2 - H), gallocatechin-(R_1 - OH, R_2 - OH), catechingallate (R_1 - GA, R_2 - H), gallocatechingallate (R_1 - GA, R_2 - OH)

The presence of the orto-dihydroxylic groups in B ring pointed to catechins capacity to scavenge the radicals [1]. Addition of the gallate-grouping in the 3rd position of C ring of the catechins increases the scavenging effect of the radicals. The major metabolites of (-)-epicatechin (EC) and of (+)-catechin, spotted within plasma after the oral administration are (-)-epicatechin-5-o-β-glucuronide and (+)-catechin-5-o-β-glucuronide [2]. These joined conjugated glucoronides showed a scavenging effect of the radicals, similar to initial substances, since the orto-dihydroxylic grouping in the 3'- and 4'- positions from B ring were not substituted. And on the contrary, metylation in 3'- position of the hydroxyl group of B ring, with the formation of 3'-o-metyl-(-)-epicatechin-5-o-β-glucuronide and 3'-o-metyl-(+)-catechin-5-o-β-glucuronide led to the decrease of super oxides' scavenging activity.

Epigallocatechin-gallate metabolites (EGCG) exercise antioxidant effects, similar to their non-conjugated compounds and which are determined by the presence of di- or trihydroxyl groups in B ring or the component gallate group. The metabolites isolated and found out in urine, (-)-5-(3',4',5'-trihydroxiphenyl)-γ-valerolacton and (-)-5-(3',4'-dihydroxiphenyl)-γ-valerolacton possess anti-oxidant properties [3]:

Catechins and polyphenols are efficient scavengers of the free radicals in various in vitro systems. The capacity of the compounds to scavenge the radicals is partially determined by the reduction potential of one electron, which is a measure of antioxidants reactivity, as donors of hydrogen or oxygen [4]. A low reduction potential proves the fact that less energy is squandered in order to donate hydrogen or electron and is an influencing factor in the antioxidant activity.

Based on table 1, we concluded that EGCG (0,43 V) and EGC (0,43 V) have [17] a lower potential than



vitamin E (0,48 V), fact suggesting that they are donors of electrons, more active than vitamin E [5]. However, vitamin C displayed a significantly lower potential of reduction (0,28 V) than all the polyphenols.

Additionally to the hydrogen or in the electron donation activity, the efficiency of the antioxidants is determined, as well by the speed of their interaction with the free radicals that are introduced into the system (constant of scavenging speed) and the stability of the formed antioxidant radical.

Taking into account the study of pulse radiolysis, which allows the comparison of the reactivity of a series of flavonoids with hydroxyl radical ($^{\cdot}OH$), super-oxide anion ($^{\cdot}O_2^-$) and azid radical ($^{\cdot}N_3$), Bors and Michel [6] conclude that the catechins are superior agents of radicals scavenging, in comparison with the monomeric flavonols and flavones, representing the most essential anti-oxidants of the red wine and tea.

Table 1

Reduction Potential and Antioxidant Activity of the Inhibitors

Antioxidant	Reduction Potential (pH 7, 20˚C)	Antioxidant Activity, mM TEAC
(-)-Epicatechin	0,57	2,4 ± 0,02
(-)-EGC	0,43	3,8 ± 0,06
(-)-ECG	0,53	4,9 ± 0,02
(-)-EGCG	0,43	4,8 ± 0,02
TeaFlavin	0,51	2,9 ± 0,08
TeaFlavin digallate	0,54	6,2 ± 0,43
Green tea (1 ppm)	-	3,8 ± 0,03
Black tea	-	3,5 ± 0,03
Vitamin E	0,48	1,0 ± 0,03
Vitamin C	0,28	1,0 ± 0,02

Therefore, the degree of polymerization that increases together with galloylation has a great influence upon the inhibitory features of the polyphenols. The polyphenolic fractions extracted from the grapes with various degrees of polymerization had a different antioxidant/antiradical and antiproliferative effect [6]. The polyphenol solutions extracted from grapes were divided with RP-HPLC into two fractions having different degrees of polymerization. The antioxidant/antiradical activity determined via DPPH test was higher for the polyphenolic fraction from grapes, composed of small monomers and oligomers, if compared to the fraction that included flavonols and procyanydin oligomers, having a great molecular mass.

The grasping activity of catechin radicals from tea was tested for different in vitro systems. The study of catechin antiradical activity setting via spectrometric method (RES) proved the efficiency of scavenging the singlet oxygen ($^1O_2$),$O_2^{-\cdot}$, $^{\cdot}OH$ and the peroxyl radical $HO_2^{\cdot}$ [7,9].

Through the radiolytic generation of species of reactive oxygen, in presence of various catechins, which are active constituents of the tea, we spotted that DNA damage, produced by these reactive particles, decreases predominantly in presence of EGCG [11]. Hence, 66% from the antioxidant activity of the green tea is determined by epigallocatechin and epigallocatechin-gallate, fact that is in compliance with the content of these compounds from the green tea (20,44% out of 26,71% of overall polyphenols). In a great part of the analyzed systems, EGCG was a better radical grasper than ECG, EGC or EC, for the reason that the trihydroxyl group from B ring and gallate component in the 3rd position of C ring amplifies the catechins antioxidant activity in various systems.

The watery extracts from Peumus boldus leaves contain catechin and alkaloids in a proportion of 37:1 and are used in hepatic infections. The antioxidant activity of these extracts is determined via $DPPH^{\cdot}$ test and is due to the catechins content [28], which is much higher than the alkaloids content.

Additionally, there were investigated the extracts obtained from aromatic and medicinal plants (sage, lavender, calendula, echinacea, etc.) through the application of $DPPH^{\cdot}$ test and ABTS [29]. These extracts, especially Salvia officinalis, have a very high free radicals scavenging activity. We noticed the correlation between extracts anti-radical activity and the total content of phenolic compounds.

Stable radicals, such as 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$) radical and $ABTS^{+\cdot}$ cation-radical (2,2- azino-bis-3- ethylbenzthiazoline-6- sulphonic acid) are used for the assessment of flavonoids in vitro antioxidant activity. The use of the Trolox equivalent antioxidant activity test (TEAA) demonstrated that the catechin and TeaFlavin are more efficient in $ABTS^{+\cdot}$ cation-radical reduction than vitamins E and C [10]. Rice-Evans and others [10] studied the total antioxidant activity (TAA) and Trolox equivalent antioxidant activity (TEAA) for the polyphenols that are contained in the green and black tea and in the red wine.

TEAA measures the concentration of Trolox solution (mM) with an antioxidant potential, equivalent to a standard concentration of the compound under study.

The authors stated that the antioxidant activity of the polyphenolic constituents of the green tea (**Fig.1**), in correlation with their content in the consecutiveness of antioxidant activity diminution is as follows: epigallocatechin (34%) ≈ epigallocatechingallate (32%) >> epicatechingallate (7%) ≈ epicatechin (6%) > catechin (1%) [10].

The catechins were more efficient in the scavenging of the DPPH radical, according to the series: EGCG≈ECG>EGC>EC [7,8]. Thus, we conclude that we obtained similar results via both methods (test with radical $DPPH^{\cdot}$ and cation-radical $ABTS^{+\cdot}$).

The overproduction of nitrogen oxide (II) and of peroxinitryl ($ONOO^-$), result of the swift interaction between $O_2^{-\cdot}$ and $^{\cdot}ON$, is well-associated with severe inflammations and might be related with the etyology and pathology of chronic diseases.

The researches carried out in vitro outlined the antioxidant potential of the polyphenols as a parameter that determines the scavenging capacity of the free radicals, as for example, super oxides radicals ($O_2^{-\cdot}$), the singlet oxygen ($^1O_2$), the hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($HO_2^{\cdot}$), nitrogen monoxide ($^{\cdot}ON$) and peroxynitrite ($ONOO^-$), which in their turn, are the causes of various pathologies. The chemical structures that contribute to polyphenols antioxidant activity, including the dihydroxy- or trihydroxy-neighbouring structure, can chelate the ions of metal, forming the complexes and preventing the creation of free radicals. This structure also allows the delocalization of the electrons, offering a higher reactivity of free radicals destruction.

There has been proved that the flavonoids, including the catechins, are efficient in scavenging $^{\cdot}NO$ in vitro [11]. The green and black teas displayed the property of scavenging $^{\cdot}NO$ in vitro, although the green tea was five times stronger than the black one [12]. The inhibition of tyrosine nitrozation was searched as a pattern of $ONOO^-$ scavenging activity by flavonoids. In this test, the catechins of the tea were much more effective than vitamin E. EGCG, ECG and

the gallic acid manifested equal properties of inhibition during the process of tyrosine nitrozation, but which were higher than EGC and EC, thus implying that the gallate component was the crucial structure in the interaction with $ONOO^-$. Additionally, EGCG manifested stronger inhibitory properties in the formation of 8-OhdG, which is the product of the interaction between the active species of nitrogen ($ONOO^-$) and DNA, than vitamin C and glutathione [13]. The exact mechanism of the inhibitory activity is unknown, but it seems that several structures are crucial in the establishment of this activity. All the catechins have the dihydroxylic group (o-3',4'-OH) in B ring that participate in the delocalization of the electron and the fixing of the radical form [14]. The gallocatechins (EGC and EGCG) have the trihydroxylic group in B ring (3',4',5'-OH), while the catechingallates (EGC and EGCG) have the gallate component esterified in the 3rd position of C ring and 3 hydroxyl groupings. The gallate component, together with the 3 hydroxyl groupings (3', 4', 5'-OH) is associated with the augmentation of the antioxidant activity [7]. The researches made in the domain of products oxidation with peroxyl radical ($HO_2^{\cdot}$) in presence of gallocatechins stated that 3',4',5'-OH grouping from B ring is the principal position in the antioxidant property of EGC and EGCG [15,16].

In different systems, the free radicals can be formed as a result of hydrogen peroxide decay in presence of various metals: Fe^{2+}, Cu^{2+}, Cr^{3+}, etc. As a consequence of H_2O_2 reduction through Fenton reaction in presence of Cr^{3+} (which is toxic and causes genotoxicity), $OH^{\cdot}$ radical is being formed:

$$Cr(III)+H_2O_2 \rightarrow Cr(IV)+OH^{\cdot}+OH^- \quad (2)$$

The hydroxyl radical formed in vivo starts DNA oxidative damage with the formation of 8-hydroxy-2-deoxyguanosine (8-OH-dG), which serves as a biomarker in this process [12].

In the result of the study, Silvia Lopez-Burillo et al. [12] concluded that the antioxidants inhibit DNA oxidative processes. Among the studied polyphenols [12], the highest inhibitory effect belongs to (-)-epigallcatechin-3-gallate (EGCG) in a concentration of 1 μM and more, and which reduces the formation of 8-OH-dG. The catechins of the tea can form stable complexes with Cu(II) and Cr(III) and as a result, there occurs the formation of $OH^{\cdot}$ radicals [13]. It was established that in the case of EGCG, $OH^{\cdot}$ radicals are separated from gallate grouping existing in complex and therefore, the pro-oxidant effect of this compound is not displayed [14].

The green and black teas are able to inhibit the oxidation of the lipoproteins induced by Cu^{2+} [13], contributing to the prevention of arteriosclerosis and other heart diseases. The inhibition of this process is determined by the fact that the polyphenols can chelate metals, decreasing the concentration of the active forms of the oxygen, which in its turn takes part in the proteins oxidation.

Anthocyanidins and catechins have been tested in vitro for the inhibitory activity on cyclooxigenase enzymes (COX) that enhance the augmentation of the carcinogen cells and influence upon the proliferation of human cancer cells [5]. It was established that cyanidin (having 3',4'-dihydroxylic groupings in B ring) has the greatest inhibitory effect among COX. The inhibitory activity decreased for delfinidin and pelargonidin, both having 3',4',5'-trihydroxylic and 4'-hydroxylic groupings in B ring. From the point of view of the link between structure and activity, the number and the position of the hydroxylic groupings within B ring of anthocyanidins influence the inhibitory activity of these compounds. For catechin, cis-, trans-isomerism and epimerization did not influence significantly the inhibitory activity of COX enzymes, but the presence of galloyl groupings in the structure of the catechins influenced their inhibitory activity on COX enzymes. Based on the results obtained during the inhibition of cancer cells proliferation under the influence of anthocyanidins and catechins, there was set that the degree of inhibition is higher for galloyl derivations of catechins [5] (gallocatechin – 95%, epigallocatechin – 100% and gallocatechingallate – 97%), while for anthocyanidins it is approximately 75%.

Certain natural colorants can be important nutritional antioxidants and their presence in the food can also reduce the risk of cancer and heart diseases [19-21]. Several researches were based on the study of such natural colorants as carotenoids, anthocyanidins and curcuminoids, that displayed antioxidant, anti-inflammatory, anti-viral, anti-cancerous properties and anti-tumour effects [22,23]. Betalains are essential natural colorants to be found in the red beet. The latest studies [24, 25] demonstrated that betalains from the beet have higher antiradical effect and antioxidant activity than carotenoids. The inhibitory concentration IC_{50} of the betanine for proteins oxidation with a lower density is superior to that of the catechin, i.e. the betanine has a higher antioxidant activity than (+)-catechin.

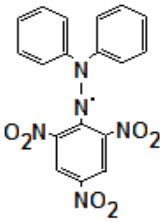
Although the betalains (betanine, betaxantine, iso-betain, amaranthine) are not flavonoids, they contain o-diphenol-monoglicosidic group and amino-grouping, manifesting very well the electro-donor properties [25]. Betalain molecules are very good electrons donors, because they contain hydroxyl (-OH) groups, amino-groupings (=NH) and tiolic (-SH) groups.

The antioxidant/antiradical activity for various betalains depends on the chemical structure and augments

together with the number of hydroxyl groups and amino-groups from molecule, while the glycosylation reduces their antiradical activity [26].

The uric acid is a grasper of free radicals within biological systems [27].

Assessment methods of antioxidant/antiradical activity of the inhibitors via DPPH· test



The chemical structure of the DPPH radical

There was studied the antioxidant/antiradical activity of various inhibitors: quercitine (Qu), resveratrol (Resv), dihydroxyfumaric acid (DFH_4), dimethylic ester of dihydroxyfumaric acid (EDMD) and extracts of grapes seeds (Eneox – enotanin non-oxidized extract, ENXIL and ENX – oxidized extracts). In this regard, we studied DPPH· test, which includes the establishment of the variation of the DPPH· radical concentration, as a result of its interaction with antioxidant components. The application of DPPH·-radical (2,2-diphenyl-1-picryl-hydrazyl) (**Fig.1**) allows to determine the antioxidant/antiradical activity of pure compounds and of extracts from vegetal products according to the variation of DPPH· concentration, which is consumed on the basis of the following mechanism:

$$DPPH^{\cdot} + RedH_2 \rightarrow DPPH\text{-}H + RedH^{\cdot} \xrightarrow{DPPH^{\cdot}} Red + DPPH\text{-}H$$

The ability of the compound to scavenge radicals is governed by their property to yield electrons or hydrogen, thus, it depends upon the potential of reduction of the antioxidants. The lower the reduction potential, the more active the antioxidant (Tab.1).

During the research, we determined the antiradical activity of the antioxidants, based on the DPPH· concentration variation in alcoholic solution (75%). The absorbance decrease was determined at λ=517 nm, at 1, 5, 10 min, during two hours. Almost in all the cases, we could obtain zero kinetic curve (without antioxidant), fact underlining DPPH· concentration variation in time. We studied DPPH· radical concentration variation for every antioxidant, depending on the reducer's concentration. Afterwards, we calculated DPPH· residual concentration (%) depending on time and we elaborated the kinetic curves. The outcomes of the kinetic behaviour of the reducer are presented in Fig.2.

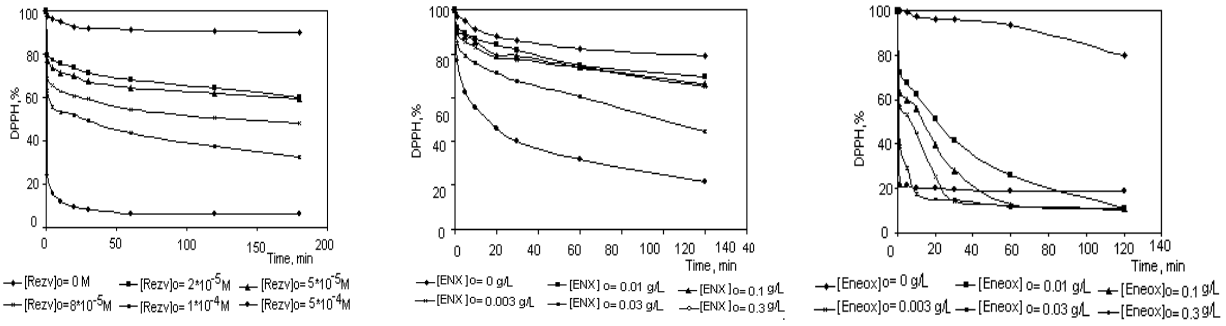
The evolution of the kinetic reactions depends on the nature of tested antioxidants. In this context, there are three types of behaviour [18]: (i) swift kinetic behaviour, in which the reaction time, t < 1 min, (ii) intermediary kinetic behaviour (5 < t < 30 min), (iii) slow kinetic behaviour, when the reaction takes place from 1 h to 6 h and more. Fig.2 shows the kinetic curves according to DPPH· percentage variation in time. We consider that Resv (a) interacts quickly. The reaction finishes within a minute, passing into a stationary state. Therefore, the resveratrol can be classified as an antioxidant that participates in reaction with the radicals, according to a swift kinetic behaviour. Other inhibitors can be classified as belonging to the intermediary kinetic behaviour type.

According to the method used in [18] the anti-radical activity was assessed based on the percentage of remained DPPH·, at the stage when the kinetic curve does not vary in time any longer. The antiradical activity (ARA) was defined as the quantity of antioxidant necessary for the decrease of DPPH· initial concentration with 50% and was called efficient concentration CE_{50} (mol/L antiox./mol/L DPPH·).

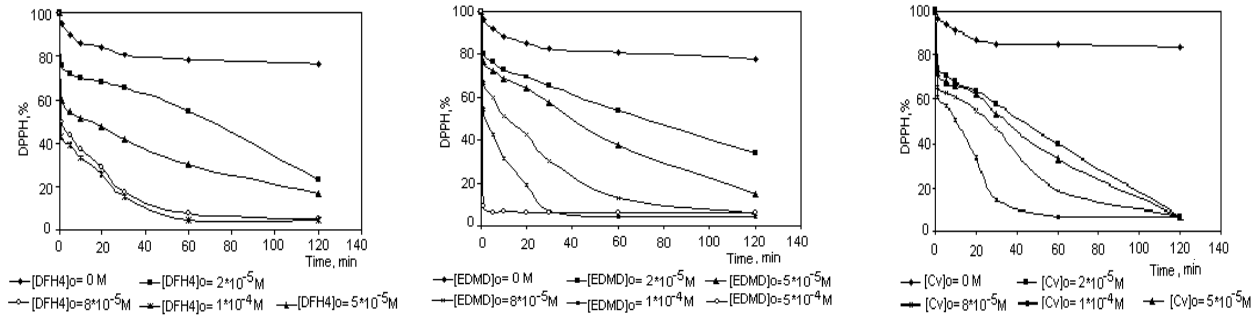
Findings and Debates

The practical study of the antioxidant/antiradical activity via DPPH· - radical test

Based on the investigational data (Fig.2) we fixed that the concentration of DPPH· is stable for all antioxidants, when setting t=120 min. Thus, taking into account all these data, we issued the diagram of concentration dependence (%) on DPPH· at 120 min, in correlation with the molar report of the antioxidant and DPPH· (Fig.3)



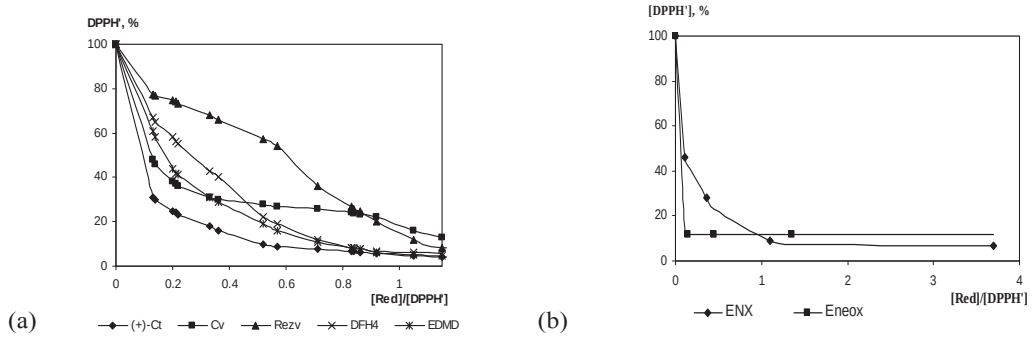
(a) (b) (c)



(d) (e) (f)

Fig. 2. The kinetic curves of DPPH˙ consumption at the interaction with: (a)-Resv; (b)-ENOXIL; (c)-Eneox; (d)- DFH_4; (e)-EDMD; (f)-Qu; in alcoholic solutions of 75%; [DPPH˙]=5·10^{-5} M

The data obtained for CE_{50} are presented in Tab.2. Based on the empirical results, we noticed that the lowest CE_{50} was characteristic for Eneox (CE_{50}^{Eneox}=0,14) while the highest - for Resv (CE_{50}^{Rezv}=1,7). Another more distinctive peculiarity of the antiradical activity of the reducers is the antiradical power (ARP), which is to be determined as the opposite mass of CE_{50} ($1/CE_{50}$). According to the data displayed in Tab. 2, ARP vary between 2,0 – 12,5, for the studied antioxidants. The lowest ARP was established for Resv (2,0), and the highest is peculiar for Eneox (12,5). ENX extract has an ARP_{Enx} equal to 6,7.



(a) (b)

Fig.3. DPPH˙ Concentration Dependence (%) on the molar report [Red]/[DPPH˙]: (a) (+)-Ct, Qu, Resv, DFH4, EDMD; (b) ENX, Eneox

The stoichiometric value equal to CE_{100}, determined via CE_{50} multiplication by two, forms the efficient concentration needed to reduce one 100% of DPPH˙. In this case, the classification of the antiradical efficiency will be incorrect, for the compounds that have a slow kinetic behaviour, because the reaction might not reach the end.

The stoichiometric value that varies within 0.2-1.0 limits is presented in Tab.2 for all the studied reducers. We also calculated the opposite amount of CE_{100} ($1/CE_{100}$), that determines the number of DPPH˙ molls from which 1 moll of reducer can be cut.

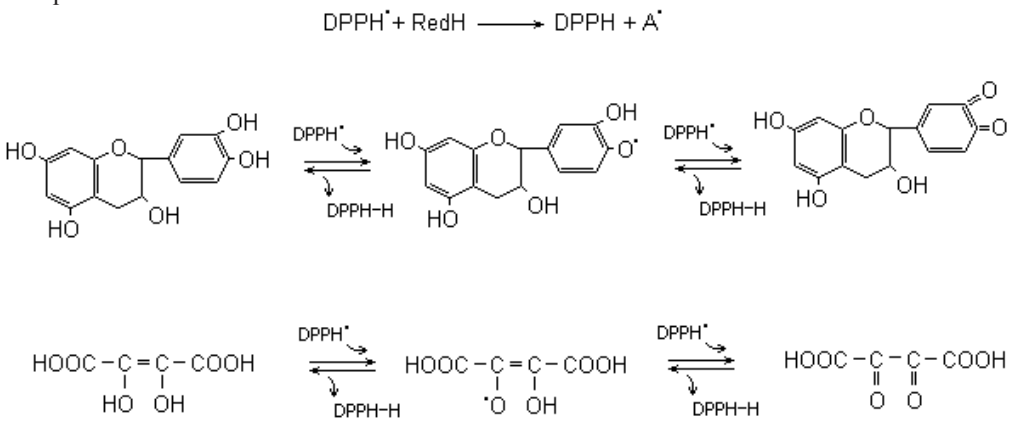
Table 2

Classification of the antiradical efficiency, in compliance with the kinetic behaviour

Kinetic Behaviour	Compounds	Efficient CE_{50} Concentration	CE_{50} x2 Stoichiometric Value	ARP ($1/CE_{50}$)	Nr. of molls DPPH˙ reduced ($1/CE_{100}$)
Fast Kinetic Behaviour (1 min)	Resveratrol	0,5	1,0	2,0	1,0
Intermediary Kinetic Behaviour (5-30 min)	ENX	0,15	0,3	6,7	3,3
	Eneox	0,08	0,16	12,5	6,25
Slow Kinetic Behaviour	Quercitine	0,1	0,2	10,0	5,0
	(+)-Catechin	0,1	0,2	10,0	5,0
	DFH_4	0,1	0,2	10,0	5,0
	EDMD	0,15	0,3	6,7	3,3

According to the data obtained in Tab.2 for the compounds that have an intermediary kinetic behaviour, $1/CE_{100}$ coincides with the number of hydrogens or hydroxyl groups, available for donation. For quercitine, (+)-catechin, $1/CE_{100}$ is approximately 2; thus, a molecule of (+)-catechin reduces 2 molecules of DPPH$^{\cdot}$, fact that corresponds to the number of hydroxyl groups participating in the reduction process, shown in the scheme below.

We calculated $1/CE_{100}$ for resveratrol, and we established that three molecules of Resv reduce one molecule of DPPH$^{\cdot}$. Taking into consideration the structure formula of the Resv, we might state that dihydroxy-grouping is lacking, i.e. there are no neighbouring dihydroxylic groups. Hence, in this case, the mechanism of radicals' removal is different from the compound that contains dihydroxylic or trihydroxylic groups. The antiradical activity of (+)-catechin is lower than that of the quercitine.



The stoichiometric value calculated for DFH_4 is 0,8, while the number of DPPH$^{\cdot}$ molls reduced by a molecule of DFH_4 is equal to 1,25, though the number of hydroxylic groupings is two.

We established the degree of inhibition (%) of DPPH$^{\cdot}$ - radical, according to the formula [29]: $ID=[A_{control} - A_{test}]\cdot 100/A_{control}$, where $A_{control}$ – is control absorbance (solution of DPPH$^{\cdot}$ without reducer) and A_{test} – is the absorbance of the tested sample (solution of DPPH$^{\cdot}$ and reducer).

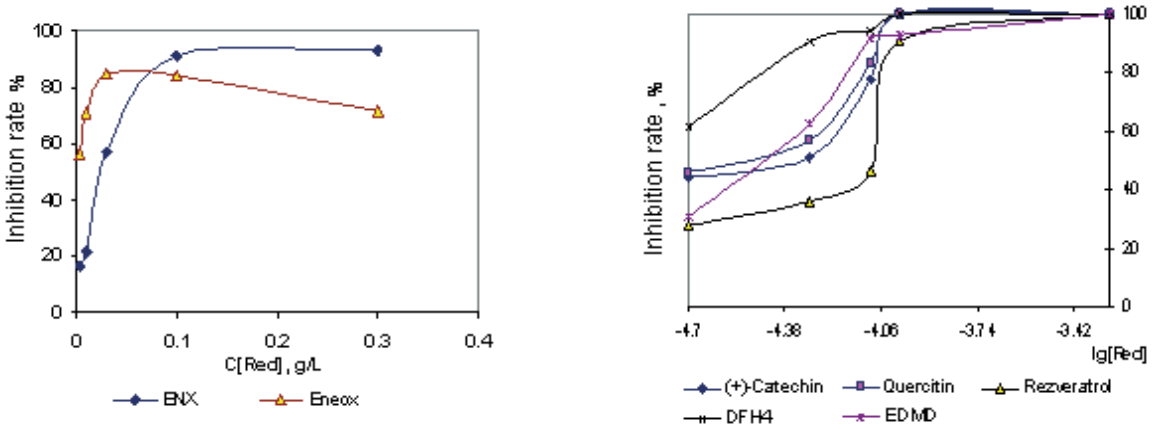


Fig. 4. ID at t=30 min depending on the concentration of the studied inhibitors

We found out that the inhibition degree (ID) increases together with the augmentation of the reducers' concentration within the interval of $2*10^{-5} – 5*10^{-4}$ M.

In case of the interaction between DPPH$^{\cdot}$ radical and DFH_4, Qu, (+)-Ct, for the interval of concentrations $2*10^{-5} – 1*10^{-4}$ M, there is a higher variation of the ID (~30-90%), and when $[Red]_o$ increases > $1*10^{-4}$ M, the ID varies insignificantly.

Dimethylic ester of dihydroxyfumaric acid and ENX (enotanin oxidized extract of grapes seeds) interacts slower with DPPH$^{\cdot}$ and ID varies from ~20% to ~70% for the interval $2*10^{-5} – 1*10^{-4}$ M, and due to ongoing increase of $[Red]_o$ to $5*10^{-4}$ M, ID reaches ~90% in respect to the control. For resveratrol, the augmentation of ID during the first interval of concentrations of Red, is the most insignificant (~20-35%), and further on at $5*10^{-4}$ M reaches 90%. In the case of Eneox (enotanin non-oxidized extract from grapes seeds), DPPH$^{\cdot}$ radical, in small quantities is wasted most rapidly. For $C_{red}=2*10^{-5}$ M there is an ID=50%. ID correlates positively with $1/CE_{100}$ calculated for these reducers (Tab. 2).

The greatest number of DPPH molecules is reduced by Eneox (6.25), further on Qu (+)-Ct, DFH_4 has $1/CE_{100}$ equal to 5, EDMD and ENX – 3.3, and Resv reduces the smallest number of DPPF molecules (1,0).

Comparing the ID of the reducers (Fig.5) for [Red]o =1*10^{-4} M, we find out that DFH_4 has the greatest ID (94%), while Resv, the lowest - (46%).

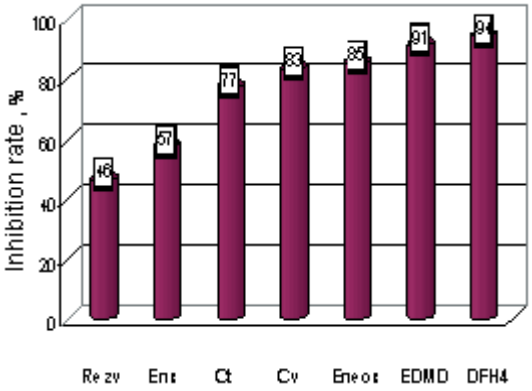
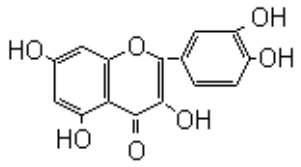


Fig. 5. ID in f (Red), for [Red]o =1*10^{-4} M

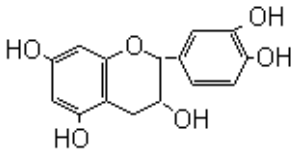
Resveratrol has a lower inhibitory activity because it is a monofenol, i.e. does not contain o-dyfenol grouping. The position of the second or third hydroxylic group is essential [30]. Those compounds, in which the second hydroxylic group is in orto- or para-position, have a higher activity than in meta-position [18].

Hence, we established that (+)-Ct, Qu have a higher grasping activity of the radicals, if compared to resveratrol, since (+)-Ct and Qu contain dihydroxylic group. Eneox extract, which contains gallocatechin, has the trihydroxylic group, that is why, its anti-radical activity is higher (EC_{50}=0.08).

If we compare (Fig.5) the antiradical activities of (+)-Ct and Qu, we notice that ID is higher for Qu (83%). The difference of the chemical structure between Qu molecule and (+)-Ct is determined by the double liaison 2,3 in conjugation with 4-oxo in C ring, responsible for the delocalization of the electrons from B ring. Similarly, groups 3 and 5-OH from A ring, together with 4-oxo group from C ring, increase the antiradical activity of Qu, if compared to that of (+)-Ct [33].



Quercitin

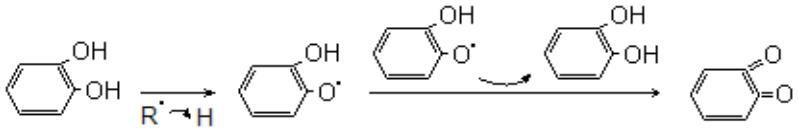


(+)- Catechin

These structural aspects of flavonols (Qu) due to which they differ from flavonones (Ct), assures a higher antiradical activity.

On one hand, the efficiency of orto- and para-diphenols is connected with aril-oxyl radical fixation by the hydrogen link or the regeneration of a diphenol molecule [30]:

Cuvelier [30] and Shahidi [31] studied the influence of the orto-metoxi substitution and deduced that ariloxyl radical is made stable through electron donation [32] and consequently, the antioxidant/antiradical efficiency increases.



Based on the experimental data presented in Fig.5, we presume that for Eneox ID is the highest, in the case of small concentrations, and together with the increase of C_{Eneox} > 1*10^{-4} M, ID decreases.

Bors [33] suggested the idea that sometimes the stability of the flavonoid ariloxyl radical is doubtful and might produce pro-oxidant effects. This effect could be explained by the formation of arbitrary liaisons that are unexpected in the case of antioxidants concentration augmentation, which in its turn decrease inhibitor's ID.

We obtained the highest degree of inhibition for DFH_4, at C_{DFH4} = 1*10^{-4} M. The presence of the –C(OH)=C(OH)– group assures a higher donation capacity of hydrogen and afterwards, the stability of the created radical. The reducing potentials of the inhibitors are inversely proportional to the donation power of electrons. The carboxyl group has a greater impact on the electrophile features.

References

[1] Nanjo F., Mori M., Goto K., and Hara Y., Radical scavenging activity of tea catechins and their related compounds, Biosci. Biotechnol. Biochem., 1999; 63:1621–1623.

[2] Harada M., Kan Y., Naoki H., et al., Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (-)- epicatechin, Biosci. Biotechnol. Biochem., 1999; 63:973–977.

[3] Li C., Lee M. J., Sheng S., et al., Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion, Chem. Res. Toxicol., 2000; 13:177–184.

[4] Halliwell B. and Gutteridge J. M. C., Free Radicals in Biology and Medicine, 3rd ed., New York: Oxford University Press, 1999.

[5] Jovanavic S. V., Steenken S., and Simic M. G., Reduction potentials of flavonoid and model phenoxyl radicals, J. Chem. Soc. Perkins Trans., 1996; 2:2497–2503.

[6] Bors W., and Michel C., Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies, Free Radic. Biol. Med., 1999; 27:1413–1426.

[7] Nanjo F., Mori M., Goto K., and Hara Y., Radical scavenging activity of tea catechins and their related compounds, Biosci. Biotechnol. Biochem., 1999; 63:1621–1623.

[8] Guo Q., Zhao B., Shen S., Hou J., Hu J., and Xin W., ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers, Biochim. Biophys. Acta, 1999; 1427:13–23.

[9] Zhao B., Guo Q., and Xin W., Free radical scavenging by green tea polyphenols, Methods Enzymol., 2001; 335:217–231.

[10] Rice-Evans C. A., Miller N. J., and G. P., Antioxidant properties of phenolic compounds, Trends Plant Sci., 1997; 2:152–159.

[11] Haenen G. R. and Bast A., Nitric oxide radical scavenging of flavonoids, Methods Enzymol., 1999; 30 :490–503.

[12] Paquay J. B., Haenen G. R., Stender G., Wiseman S. A., Tijburg L. B., and Bast A., Protection against nitric oxide toxicity by tea, J. Agric. Food Chem., 2000; 48:5768–5772.

[13] Fiala E. S., Sodum R. S., Bhattacharya M., and Li H., (-)-Epigallocatechin gallate, a polyphenolic tea antioxidant, inhibits peroxynitrite-mediated formation of 8–oxodeoxyguanosine and 3–nitrotyrosine, Experientia, 1996; 52:922–926.

[14] Rice-Evans C. A., Miller N. J., and Paganga G., Structure- antioxidant activity relationships of flavonoids and phenolic acids, Free Radic. Biol. Med., 1996; 20:933–956.

[15] Valcic S., Muders A., Jacobsen N. E., Liebler D. C., and Timmermann B. N., Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (-)-epigallocatechin gallate with peroxyl radicals, Chem. Res. Toxicol., 1999; 12:382–386.

[16] Valcic S., Burr J. A., Timmermann B. N., and Liebler D. C., Antioxidant chemistry of green tea catechins. New oxidation products of (-)-epigallocatechin gallate and (-)-epigallocatechin from their reactions with peroxyl radicals, Chem. Res. Toxicol., 2000; 13:801–810.

[17] Jane V. Higdon and Balz Frei, Tea Catechins and Polyphenols: Health Effects, Metabolism, and Antioxidant Functions. Linus Pauling Institute, Oregon State University, Corvallis, OR 97331.

[18] W. Brand-Williams, M. E. Cuvelier and C. Berset, Use of a free radical method to evaluate antioxidant activity. Lebensm, u, Technol, 28, 25-30(1995).

[19] Pszczola, D. E. Natural colors: pigments of imagination. *Food Technol.* 1998, 52, 70-76.

[20] Kritchevsky, S. B. /3-Carotene, carotenoids and the prevention of coronary heart disease. *J. Nutr.* 1999, *129,* 5-8.

[21] Mazza, G. Health aspects of natural colors. In *Natural Food Colorants Science and Technology',* Lauro, G. J., Francis, F. J., Eds.; Marcel Dekker: New York, 2000; pp 289-314.

[22] Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y. C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and antinflamatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* 1999, *62,* 294-296.

[23] Espin, C. J.; Soler-Rivas, C.; Wichers, H. J.; Garcia-Viguera, J. Anthocyanin-based natural colorants: a new source of antiradical activity for foodstuffs. *J. Agric. Food Chem.* 2000, *48,* 1588- 1592.

[24] Pedreno, M. A.; Escribano, J. Studying the oxidation and the antiradical activity of betalain from beetroot../. Biol. Ediic. 2000, 35, 49-51.

[25] Kanner, K.; Harel, S.; Granit, R. Betalains—A new class of dietary cationized antioxidants. J. Agric. Food Chem. 2001, 49, 5178-5185.

[26] Yizhong Cai, Mei Sun and Harold Corke, Antioxidant activity of Betalains from Plants of the Amaranthaceae.

[27] E. Abuin, E. Lissi, P. Ortiz and C. Henriquez Uric Acid Rreaction With DPPH Radicals at the micellar interface.

[28] G. Schmeda, J.A. Rodriguez, C. Theoduloz, S.L. Astudillo, G.E., Feresin and A. Tapia, Free- radical Scavengers and Antioxidants from Peums boldus Mol., Free-radical research, 2003, Vol 37(4), pp. 447-452.

[29] Son, S.; Lewis, B. A. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: Structure-activity relationship. *J. Agric. Food Chem.* 2002**,** 50, 468-472.