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Synergistic, Additive and Antagonistic Interactions of Some Phenolic Compounds and Organic Acids Found in Grapes

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Abstract

The antioxidant interactions between several natural phenolic and non-phenolic compounds (catechin, quercetin, rutin, resveratrol, gallic acid and ascorbic acid) and organic acids (tartaric, citric and dihydroxyfumaric acids) were studied using the DPPH method. Main additive and antagonistic interactions have been found for the combinations of catechin, quercetin, resveratrol and gallic acid with tartaric and citric acids; such behavoir can be due to the enhanced stability of the phenolic compounds in acidic media. Rutin and ascorbic acid showed good synergistic effects with tartaric and citric organic acids, which could be due to the polymerization processes in the case of rutin and the change in the mechanism of action in the case of ascorbic acid. In combination with dihydroxyfumaric acid, the mixtures showed dose–dependent synergistic, additive, or antagonistic antioxidant interactions. Good synergistic effects were observed for the binary mixtures of dihydroxyfumaric acid with ascorbic acid, catechin, and rutin.

Keywords: antioxidant interactions; synergistic interactions; additive interactions; antagonistic interactions; phenolic compounds; organic acids.

1. Introduction

Antioxidant interactions (AI) have been investigated more intensively in the last twenty years. The interest in AI is justified by the scientific intention to understand the natural processes, but also by the real need to improve the antioxidant activity of natural compounds used in the food, medical, pharmaceutical and other industries, by finding beneficial combinations between antioxidant and non-antioxidant compounds. Until now, synergistic, additive, and antagonistic AI between natural compounds have been declared.[1] Some explanations and hypotheses on the mechanism of mutual interaction of involved compounds have been offered, and imply (1) the regeneration processes, (2) formation of antioxidants` intermolecular complexes, dimers or adducts, and (3) complementary effects that presume the effect of solvent, pH, concentration and solubility.[1,2]

Organic acids such as tartaric and citric are common acids, non–antioxidant compounds, found in large amounts in many fruits, including grapes.[3,4] Although they are not free radical scavengers,[5,6] their influence on the antioxidant activity of natural reducing compounds has already been demonstrated. The authors found that the combinations of various natural radical scavengers with organic acids have synergistic AI.[5–9] On the other hand, the interactions between grape phenolic compounds were found to be antagonistic, which is due to the polymerization processes and the decrease in the number of electron donating groups.[10–14]

The compounds' concentrations showed to be equally important for antioxidant activity and AI.[12,14,15] According to the reported data,[15–19] it is generally believed that stronger synergistic effects can be obtained when compounds are used at concentrations found in nature (in this case, in grapes),[7,12,20–25] since multicomponent systems similar in composition and concentration to those found in food have multiple mechanisms of action and can inhibit oxidation at many different stages.[26]

Based on this, the present study aims to investigate the influence of different concentrations of common organic acids, namely tartaric and citric acids, on the antioxidant activity of phenolic and non-phenolic compounds found in grapes: catechin, quercetin, rutin, resveratrol, gallic acid and ascorbic acid. In addition, the AI of the above compounds was studied with the natural organic acid, dihydroxyfumaric acid, which has potent antioxidant activity,[6,27] and is known to be important for the "glioxylate scenario"[28,29]. Experimental data were obtained by the DPPH method, readily available and widely used antioxidant assay, so that the results could be easily compared with literature data.

2. Exprimental

2. 1. General

Quercetin dihydrate (QUE), (+)-catechin (CAT), (+)-rutin trihydrate (RUT), *trans*-resveratrol (RES), L-ascorbic acid (AA), dihydroxyfumaric acid hydrate (DHF), L-(+)-tartaric acid (TA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (Germany), gallic acid (GA), citric acid (CA) and 96% ethanol (EtOH) were purchased from MicTan (Republic of Moldova).

Absorbance measurements were recorded on a Lambda 25 UV/VIS spectrophotometer (Perkin Elmer), at 20 ˚C, using 10 mm quartz cuvettes.

The pH was measured on a HANNA HI 121 pH-meter, using 96% EtOH as solvent.

2. 2. Preparation of Standard Solutions and Mixtures of Phenolic Compounds and Organic Acids

Standard solutions of AA (1.4 mM), CAT (1.2 mM), GA (1.4 mM), QUE (1.0 mM), RUT (1.0 mM), RES (0.5 mM), DHF (2.0 mM), TA (40.0 mM) and CA (40.0 mM) were prepared in 96% EtOH. For a better dissolution, some of the samples were sonicated in the ultrasonic bath for 3 – 5 min. For the determination of the Efficient Concentration (EC_{50}) of single compounds, different concentrations of CAT, GA, QUE, RUT, RES, AA, and DHF ranging from 50 μM to 1000 μM, and different concentrations of TA and CA ranging from 0.2 mM to a maximum of 20 mM were prepared by dilution from stock solutions, using 96% EtOH

To study AI, the given concentrations of CAT, GA, QUE, RUT, RES, and AA (ranging from 50 μM to 1000 μM) were mixed with three concentrations of TA or CA, found in grapes and wines (16×10^{-4} N, 160×10^{-4} N, 800×10^{-4} N), and with three concentrations of DHF (2×10^{-4} N, 4×10^{-4} N, 8×10^{-4} N). This approach was described by LoScalzo in an attempt to clarify the influence of some organic acids on the antioxidant activity of ascorbic acid.[5]

2. 3. DPPH Free Radical Scavenging Activity

The concentration of DPPH in 96% EtOH was verified daily though the calibration line and was around 0.03 g/L (Absorbance = 1.000 ± 0.020 a.u.). The absorption maximum of DPPH was found to be at 517 nm with a molar extinction coefficient ε, of 11858 ± 16 M^{-1} cm^{-1}.

The antioxidant activity of individual compounds and mixtures was estimated according to procedure described previously.[30] To 3.9 mL of free radicals, 0.1 mL of the prepared samples containing the tested compounds was added. Absorbance at 517 nm was recorded when the reactions reached equilibrium, which was after 30 min for GA, AA, DHF, TA, and CA, and after 60 min for QUE, CAT, RUT, and RES. The blank reference cuvette contained 96% EtOH. All measurements were performed at least in triplicate.

2. 4. Data Analysis

Following the approach reported by Brand-Williams et al,[30] the antioxidant activity of the compounds or mixtures of compounds tested was expressed as an EC_{50} value, defined as the concentration required to annihilate 50% of the radical and expressed as mole of antioxidant per mole of $DPPH^{\bullet}$ (mole AOX/mole DPPH). This parameter is inversely related to the antioxidant capacity of the compound studied, with lower EC_{50} values indicating higher antioxidant activity.

In order to determine EC_{50} parameter, the percentage of remaining $DPPH^{\bullet}$ (%DPPH rem) at the steady state was calculated according to equation 1, and the results obtained for each sample were plotted against the mole AOX/mole DPPH ratio to determine the EC_{50} value.

$$\%\text{DPPH rem} = \left(\frac{A_{sample}}{A_{control}}\right) \times 100 \qquad (1)$$

The A_{sample} corresponds to the absorbance of the sample at steady state and $A_{control}$ corresponds to the absorbance of the sample at time zero. Because the EC_{50} is related to the stoichiometry of the reaction, results presented as EC_{50} values are more accurate and free of error; in addition, these results are easier to compare with literature data.

The percentage of inhibition (%Inhibition) was obtained using equation 2, and was further used to determine the AI type.

$$\%\text{Inhibition} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \qquad (2)$$

From equation 2, A_{sample} is the absorbance of the sample at steady state and $A_{control}$ is the absorbance of the sample at time zero.

The AI effect of a mixture was calculated from the ratio between the experimental value of the percent inhibition of the mixture ($\%I_{mixture}$) and the theoretical value ($\%I_{theoretical}$,[9] (equation 3):

$$\text{AI} = \left(\frac{\%I_{mixture}}{\%I_{theoretical}}\right) \qquad (3)$$

Where

$$\%I_{theoretical} = \%I_A + \%I_O - \left(\frac{\%I_A \times \%I_O}{100}\right) \quad (4)$$

$\%I_A$ and $\%I_O$ represent the percent inhibition of antioxidants and organic acids, respectively, tested in reaction with DPPH$^\bullet$ alone (equation 4).

Therefore, a synergistic effect is found when the AI > 1; if AI = 1, then the interaction is additive; and an AI < 1 reveals an antagonistic effect.

The data obtained were analyzed with ANOVA and Student's *t* tests to evaluate the statistical significance of the difference between the means using the Microsoft Excel programme. A *p* value of 0.05 was considered significant.

3. Results and Discussion

3. 1. Determination of the EC_{50} Values

The literature frequently reports the use of the DPPH method to study the antioxidant activity of individual compounds and to determine the type of AI between natural compounds.[8,9,13,31] The DPPH$^\bullet$ can be scavenged through both HAT and SET mechanisms,[32] depending on the reaction conditions. This suggests that DPPH$^\bullet$ takes either an electron or an H atom from the antioxidant to be neutralized.

Phenolic compounds possess good antioxidant activity against various free radicals[11–13,31] due to the presence of functional groups and conjugated double bounds. Table 1 shows the EC_{50} obtained for individual compounds in the reaction with DPPH$^\bullet$, as well as the EC_{50} values for the combinations of AA, CAT, QUE, RUT, RES and GA with organic acids – DHF, TA and CA. As mentioned earlier, the lower is the EC_{50} for a compound, the higher is the antioxidant activity. On this basis, GA is the best radical scavenger under these reaction conditions, followed by the other compounds in the order: QUE < CAT = DHF < AA = RUT < RES.

Similar results have been reported by several authors;[11,13,31,33] the few differences are attributed to the use of different solvents, which have been shown to have a significant effect on the mechanism of action of antioxidants.[6]

The stilbene RES possesses good antioxidant capacity against reactive oxygen species, especially against superoxide anion.[34] However, in the reaction with DPPH$^\bullet$, RES demonstrates low antioxidant activity, which is confirmed by previous studies.[11,13,31,33] The compound DHF, which in this study was addressed as an organic acid, possesses good antioxidant activity, with an EC_{50} = 0.18, which means that DHF in a multicomponent system can affect the overall antioxidant activity even at low concentration. Contrary to DHF, the organic acids TA and CA showed insignificant radical scavenging activity – even at high concentrations, TA and CA are capable of scavenging only 3% of free radicals. Similar results have been reported by others.[5,8,9]

As expected, the presence of organic acids in the solutions lowers the pH to a more acidic value (Table 2). Such pH values are representative of wines, natural juices or fruits.[4,35]

Table 2. pH values for each concentration of organic acids (TA, CA and DHF) used in experiments. EtOH was used as solvent.

		Organic acids			
		TA	CA	DHF	96% EtOH
Concentrations					6.58
	16×10^{-4} N	4.43	4.42		
	160×10^{-4} N	3.75	3.89		
	800×10^{-4} N	3.10	3.58		
	2×10^{-4} N			4.04	
	4×10^{-4} N			3.86	
	8×10^{-4} N			3.64	
	Student's *t* test	*	*	*	*

Significant difference ($p < 0.05$) to value 6.58. *p* values were calculated using one-sample Student's *t* test.

Table 1. EC_{50} values for individual antioxidant compounds and for their combinations with organic acids.

				Antioxidant compounds						
				AA	CAT	QUE	RUT	RES	GA	DHF
		Reaction time, min		**30**	**60**	**60**	**60**	**60**	**30**	**30**
EC_{50} (mole AOX/mole DPPH)		No organic acid		*0.24±0.00*	*0.18±0.02*	*0.15±0.00*	*0.24±0.00*	*1.15±0.03*	*0.06±0.00*	*0.18±0.01*
	Organic acids	TA	16×10^{-4} N	0.22±0.01	0.19±0.00	0.16±0.01	0.25±0.01	1.22±0.01	0.06±0.00	
			160×10^{-4} N	0.22±0.01	0.18±0.01	0.18±0.00	0.26±0.00	0.98±0.00	0.06±0.01	
			800×10^{-4} N	0.23±0.00	0.18±0.01	0.18±0.00	0.27±0.01	1.78±0.00	0.06±0.01	
		CA	16×10^{-4} N	0.23±0.00	0.18±0.00	0.17±0.00	0.25±0.00	1.59±0.02	0.06±0.00	
			160×10^{-4} N	0.23±0.00	0.21±0.02	0.18±0.03	0.26±0.00	1.14±0.00	0.05±0.00	
			800×10^{-4} N	0.24±0.01	0.18±0.00	0.18±0.02	0.29±0.01	2.83±0.04	0.06±0.00	
		DHF	2×10^{-4} N	0.20±0.00	0.13±0.00*	0.14±0.01*	0.21±0.00*	1.32±0.01	0.09±0.00	
			4×10^{-4} N	0.17±0.00	0.12±0.00*	0.13±0.00*	0.17±0.00*	2.19±0.04	0.08±0.01	
			8×10^{-4} N	0.09±0.01	0.09±0.00*	0.09±0.00*	0.09±0.02*	1.01±0.01	0.06±0.00	

Data are presented as means ± deviation (n ≥ 3). * Significant difference ($p < 0.05$) calculated using one-sample Student's *t* test.

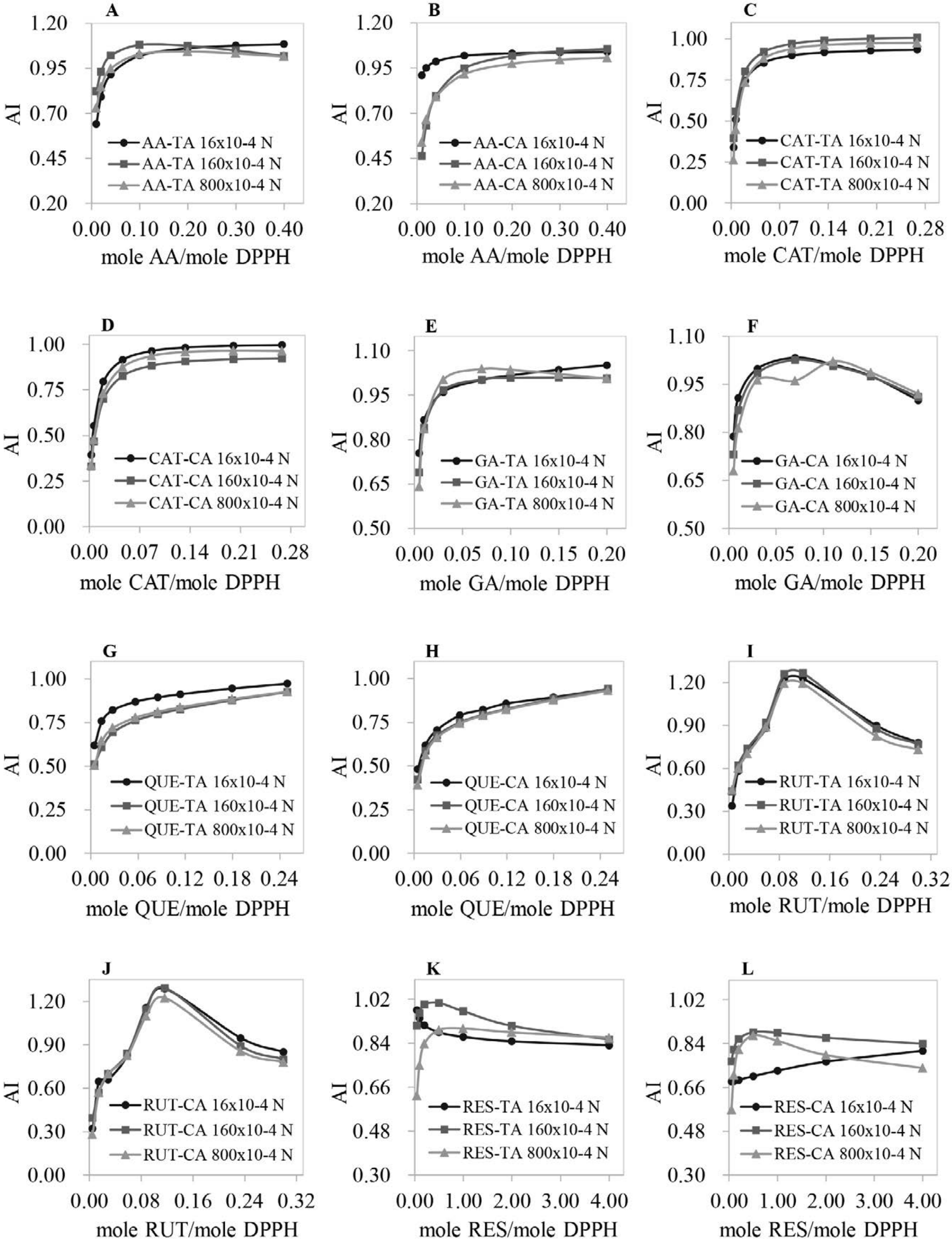

Figure 1. AI of AA and phenolic compounds in the combination with different concentrations of TA (A, B, C, D, E, F) and different concentrations of CA (G, H, I, J, K, L). Data are presented as mean values (n ≥ 3).

Different results were observed by adding organic acids – DHF, TA or CA, to the reaction mixtures between the antioxidant compound and DPPH$^•$. The decrease in EC_{50} values, which can be interpreted as cooperative activity of two compounds that increases the overall antioxidant activity of the mixture, was registered for combinations of AA – DHF and AA – TA, and for polyphenols CAT, QUE and RUT with DHF. On the contrary, the addition of TA or CA to solutions of phenolic compounds have a negative impact on the EC_{50} values of the mixtures and in most cases, leads to an increase in this parameter.

The concentration of organic acids showed to be important for the antioxidant activity of the tested mixtures, however a prevalent tendency cannot be reported at this point. For example, increasing the DHF concentration from 2×10^{-4} N to 8×10^{-4} N the EC_{50} for AA, CAT, QUE and RUT decreases, but it becomes higher for RES and GA.

Concerning the other organic acids, the presence of TA or CA in concentrations of 16×10^{-4} N and 160×10^{-4} N has a slightly positive effect on the antioxidant activity of AA, but a negative or no effect in the mixtures with CAT, QUE, RUT and for the majority of the combinations with GA. Except for the presence of 160×10^{-4} N of both TA and CA in the reaction mixtures of RES and DPPH$^•$, the other two concentrations of organic acids produce significant increase in the EC_{50} values, especially for the combination of RES with 800×10^{-4} N of CA.

The investigation of different concentrations of DHF, TA, and CA shows that the acidic environment in grapes and grape products can positively or negatively affect the antioxidant activity of phenolic and non-phenolic compounds; it also shows that not only the acidic environment is crucial, but also the intrinsic properties are important. Although the variation of EC_{50} values shows that the presence of organic acids affects the antioxidant activity of phenolic compounds and AA, these data are insufficient to classify the tested mixtures according to the type of AI – synergistic, additive, or antagonistic; therefore, further calculations are needed.

3. 2. AI Between Phenolic Compounds and Organic Acids TA and CA

Among all three types of AI, synergistic interactions are the most advantageous, therefore more intensively investigated. Recently, authors have demonstrated the importance of the non-antioxidant substances such as organic acids, glucose, etc., for the antioxidant activity of naturally occurring bioactive compounds,[5,7–9] and for the quality of the food products.[36–38] The concentrations of the compounds have been shown to be equally important for AI, so that different molar ratios between the same compounds can lead to synergistic, additive, or antagonistic effects.[9,14,15,39–41] In this study, the utilization of three concentrations of organic acids was important for evaluation of their impact on the antioxidant activity of the phenolic and non–phenolic natural compounds. In addition, the importance of the concentration of the antioxidants tested was evaluated by applying different concentrations of phenolic compounds or AA as depicted in Figure 1.

The AI between AA and other organic acids

R. LoScalzo[5] and Piang-Siong et al.,[9] investigated the interaction of AA with organic acids in alcoholic solution, and found significant synergistic effects. Similarly, our results revealed that at certain concentrations, TA and CA ameliorate the antioxidant activity of AA. Data reported in Figure 1 (cases A and G) show that TA has a better influence than CA, being observed six combinations of AA – TA with synergistic effects, and only one combination of AA – CA with the same effect. In both cases, smaller concentrations of organic acids, namely 16×10^{-4} N and 160×10^{-4} N, cause the enhancement of antioxidant activities, being registered synergistic effects of 1.08 for the mixture AA – TA, and 1.06 for AA – CA. Equally important is the concentration of AA. Strong antagonistic effects have been noticed at lower concentrations of AA, and by increasing the AA's content, the AI values rise, reaching the additive and synergistic effects. The notable antagonism, in the range of 0.46 – 0.79, characteristic for lower AA concentrations, and the synergistic effects found at higher AA concentrations, emphasize the importance of the molar ratios in which both natural compounds are mixed.

The enhancement of the antioxidant activity of AA in the presence of organic acids may be due to the action mechanism of this free radical scavenger. The ionization of AA is not supported in this media because of the high amount of ions of TA or CA present in the solution. Consequently, the SPLET (sequential proton loss electron transfer) mechanism is inhibited, and the HAT (hydrogen atom transfer) mechanism becomes operative for DPPH$^•$ annihilation. The AA is known to be efficient in HAT reaction by donating two H atoms to the radical species.[42] R. LoScalzo suggested that a low pH can contribute to the slow regeneration of AA, thus justifying the enhancement of the antioxidant activity.[5]

The AI between CAT and organic acids

In the presence of organic acids, the phenolic compound CAT shows a progressive evolution of the AI values from strong antagonistic effects to additive effects (Figure 1, cases B and H). Samples containing small concentrations of CAT and TA or CA, demonstrated antagonistic interactions in the range of 0.33 – 0.93; the increase of CAT's concentration generated additive AI. Contrary to the example of AA's interactions with organic acids, the change in the TA or CA content does not affect considerably the antioxidant activity of CAT. Similar antagonistic interactions were reported by Zhang et al.,[43] who investigated the influence of organic acids on the antioxidant activity of phenolic compounds from Zhenjiang aromatic vinegar.

The reaction of CAT and DPPH$^•$ was previously studied in alcohols.[44–47] It was demonstrated that there is the possibility of (1) covalent adduct formation between the free radical and the oxidized form of CAT, and (2) the chance of polymerization reaction, which proved to be less effective for DPPH$^•$ scavenging.[46] The two pathways depend on the polarity of the solvent and on the flavanol/DPPH$^•$ ratio.[46] The addition of organic acids to the reaction mixture can affect significantly the reactivity of CAT, and finally the total antioxidant activity. Catechins showed to be more stable at low pH,[48,49] their antioxidant activity being 10 times higher at neutral pH than at acidic pH.[50] In acidic environments like those created by the addition of TA or CA (Table 2), the oxidation rates of the phenolic compounds increase. As a consequence, the ability of CAT to donate electrons and to scavenge DPPH$^•$ decreases, thus, only additive and antagonistic interactions are registered. A pH dependent change of the antioxidant activity of polyphenols was noticed by others;[51,52] the greater reactivity of phenolic compounds at high pH was attributed to the rapid electron transfer from the phenolate ion to the reactive species.

According to data, polar solvents maintain the SPLET mechanism of antioxidant action of phenolic compounds,[46,53] because these solvents accept protons from the phenol forming the phenolate anion followed by the electron transfer to the reactive species. The presence of the acid ions in the reaction mixture suppresses the deprotonation, and, by this, the SPLET mechanism, so the electron donor will be the parent molecule.[54] At low pH, CAT is expected to be oxidized via the ET-PT (electron transfer – proton transfer) mechanism, which implies the electron abstraction from the neutral molecule followed by the release of a proton.[55]

The diminution of the antioxidant activity of CAT in the presence of TA or CA can also be justified by the fact that low pH conditions might enhance CAT loss on account of its polymerization and condensation.[49] The investigation of the condensed tannins proved that under acidic conditions two competing reactions occur: (1) the polymeric or oligomeric chain can be degraded to their monomers and (2) the flavonoid units can condense.[56] The processes of hydrolysis, condensation and heterocyclic ring opening at low pH are described in the literature as common reactions for tannins.[57] Studies showed that the formation of oligomers of CAT or QUE is due to the cleavage of the interflavonoid bond, and can also be acid–catalyzed.[58,59] Such opposite and competing reactions are characteristic for wine systems, where the presence of organic acids promotes both polymerization and hydrolysis of phenolic compounds.[60]

The AI between GA and organic acids

The AI of GA with TA or CA is characterized by additive and antagonistic effects (Figure 1, cases C and I). In the presence of TA, the AI values are lower at small concentrations of GA, but augment to additive effects at bigger GA/DPPH molar ratios. The only synergistic effect of 1.05 has been registered for the GA/DPPH molar ratio of 0.20 and the 16×10^{-4} N of TA. The samples containing GA and CA showed an ascending tendency of AI values (maximum AI value of 1.03) followed by a descending one, starting from 0.15 GA/DPPH molar ratio. In this case, no synergistic effects have been noticed. These results are supported by similar AI between GA and organic acids that have been reported by Piang-Siong et al.[9] The fact that the increase of TA or CA concentrations does not cause major variations of the AI values proves that the acidity, regardless of its magnitude, has the same effect on the antioxidant activity of GA. One exception is the situation with the smallest concentration of TA, where the reaction keeps a positive tendency.

According to the EC_{50} values (Table 1) and to the AI values of GA with organic acids, it can be inferred that TA or CA have slightly negative or no effect on the GA's antioxidant activity. Therefore, it can be supposed that GA operates efficiently in acidified ethanolic solutions through the HAT mechanism. The carboxyl group of GA along with the phenolic OH tends to deprotonate in ethanol, but the presence of the ions of TA or CA suppresses this process, therefore, likewise the example of CAT, the SPLET mechanism is hindered. Hydrogen transfer mechanism becomes operative for GA in this environment. This assumption is in agreement with the data from DFT calculations,[61–63] which demonstrate that GA is an excellent free radical scavenger by H atom donation.[18,64]

The AI between QUE and organic acids

The interaction between different concentrations of QUE and TA or CA demonstrated only antagonistic effects in the range of 0.50 – 0.94 and 0.39 – 0.94, respectively, except for one additive interaction of QUE – TA with the value 0.97 (Figure 1, cases D and J). Figure 1D is clearly indicating that at larger TA concentrations the antagonistic effects are stronger. This fact and the persistence of the antagonistic interactions independently of the TA or CA content underline the idea of diminution of the free radical scavenging activity of polyphenols in acidic environments.[46,51,52,54] Similar to catechins,[48,49] QUE is more stable at low pH, and therefore less susceptible to oxidation.

The AI between RUT and organic acids

The flavonoid RUT manifests a specific behavior in the presence of organic acids. At lowest concentrations of the polyphenol, strong antagonistic effects, in the range of 0.29 – 0.92, can be noticed, (Figure 1, cases E and K). However, at the 0.09 and 0.12 RUT/DPPH molar ratios, significant synergistic interactions, namely 1.10 – 1.29, are observed, which represent the highest AI values in this series of experiments. The synergistic effects of these two RUT/DPPH molar ratios decrease slightly with the increase of the RUT content. In the same time, the AI between RUT and TA or CA appears to be independent from the concentration of organic acids. These results indicate that, in the case of RUT and TA or CA combinations, the synergistic effect relies mainly on the concentration of polyphenol, being independent of the acid's content. Still, the presence of the organic acids in the reaction mixture is essential for the synergistic effect to occur, as long as the antioxidant activity of RUT is smaller without TA or CA (data not shown).

RUT is the only phenolic compound in this series of experiments to demonstrate such distinctive behavior characterized by a sharp peaking of the AI at 0.09 and 0.12 RUT/DPPH molar ratios. This effect can be caused by the presence of rutinose in the RUT structure, which is absent in the molecule of other tested phenolics. More than that, QUE and RUT have the same aglycon structure, however, QUE in combination with TA or CA manifested only additive and antagonistic effects. Data[45] show that the structural differences in the C ring – the C3 hydroxyl group is present in QUE, but is glycosylated in the case of RUT, affect considerably the antioxidant activity.

Also, the change from antagonistic to synergistic AI may be due to the concentration of reactants, as it has been found for different CAT/AA ratios.[15] The concentration of AA affects CAT's behavior, supporting the formation of different structures, including CAT dimerization to the procyanidin structures.[15] This could also be the situation for the RUT – TA or CA synergistic effect, however, for this example, the concentration of organic acids appears to be insignificant – matters only their presence. This assumption is supported by other findings that describe the formation of dimers and polymers from CAT, QUE and RUT in acidic conditions.[58,59] On the other hand, it should be admitted that some polymerizations and adduct formations may be determined by the flavanol/DPPH$^\bullet$ ratio, as previously demonstrated,[44–46] along with the formation of new structures with antioxidant properties.

The AI between RES and organic acids

The combination of stilbene RES with organic acids shows mainly antagonistic effects (Figure 1, cases F and L). The tendencies described by the AI values are different for each concentration of organic acids. The strongest antagonistic interactions are noticed for the concentration of TA or CA of 16×10^{-4} N, and the less antagonistic effect – for the mixtures containing 160×10^{-4} N. The negative effect of organic acids on the RES antioxidant activity is in agreement with the data reported by Shang et al.[54] The authors showed that in acidic media the ionization of the RES is suppressed, along with the SPLET mechanism of action, therefore, the antioxidant activity is reduced.[54]

Based on data from Figure 1 and on the results described in the literature, it can be concluded that organic acids can influence the antioxidant activity of phenolic compounds by either determining their mechanism of action, or by inducing polymerization or cleavage of the intermolecular bonds of the formed oligomers. Authors[11,13,14,39,65] demonstrated that combination of natural polyphenols shows mainly antagonistic effects, because of their tendency to combine themselves through polymerization, thus decreasing the availability of the hydroxyl groups. We suggest that the addition of organic acids to the reaction mixtures, followed by the increase of acidity, could prevent polymerization by intermolecular bonds break and would maintain a high degree of low complexity structures, and a standing number of electron donating groups. This hypothesis is supported by the fact that the natural environment of the antioxidants from fruits and vegetables is characterized by high content of organic acids and a relatively high acidity, comparable with that created by addition of TA, CA or DHF (Table 2). Also, the antioxidant power of extracts from fruits and vegetables that proved to be stronger than to the sum of the antioxidant activities of individual compounds[11,14] can be argued by the presence of less active compounds from natural sources, like organic acids.

3. 3. AI Between Phenolic Compounds and Dihydroxyfumaric Acid

The DHF was for the first time discovered in 1994 by Fenton[66] in his attempt to oxidize TA in the presence of hydrogen peroxide and iron. Lately, the DHF was intensively studied from the perspective of the "glyoxylate scenario".[28,29,67] These investigations have been focused on the propounded idea that DHF could be the central starting materials of chemical constitution for primordial metabolisms – or, the building-blocks for the biogenic molecules.[28,29,67,68]

From the point of view of its occurrence, DHF is widespread in natural products, being a constituent of the reductive citric acid cycle, and therefore a direct precursor of amino acids[67] and a constituent of the cycle of dicarbonic acids – the Baround' cycle of tartaric acid and its intermediate products transformation to oxalic acid.[69,70] DHF is found in wines as reaction product of the TA oxidation by hydroxyl radicals;[71] also, it is added in wines for the enhancement of the quality parameters.[72,73]

The AI between AA and DHF

The DHF showed to be a strong DPPH$^\bullet$ scavenger, its antioxidant activity being comparable with and even stronger than that of the AA, in terms of the kinetics and stoichiometry.[6,27] Starting from this context, we assumed that in combination with phenolic compounds DHF would behave similarly to AA. The synergistic interactions between phenolic compounds and AA have been described in the past few years,[12,15,21,74] authors suggesting that the synergistic effects are due to the regeneration of the polyphenols by the AA. In our attempt to clarify the type of AI between DHF and natural phenolic compounds similar outcomes have been expected.

Previously, we reported data on the synergistic and antagonistic interactions between DHF and AA in 98% EtOH and in wine matrix.[6] This study was performed using the Stopped-flow method, which enabled us to gather data only for the first 2 sec of the reaction. Comparing our results with the data reported in the literature,[27,31] we concluded that, after 2 sec of interaction of AA or DHF alone or in combination against DPPH$^{\bullet}$, the reaction is incomplete and requires further investigations. Figure 2A shows

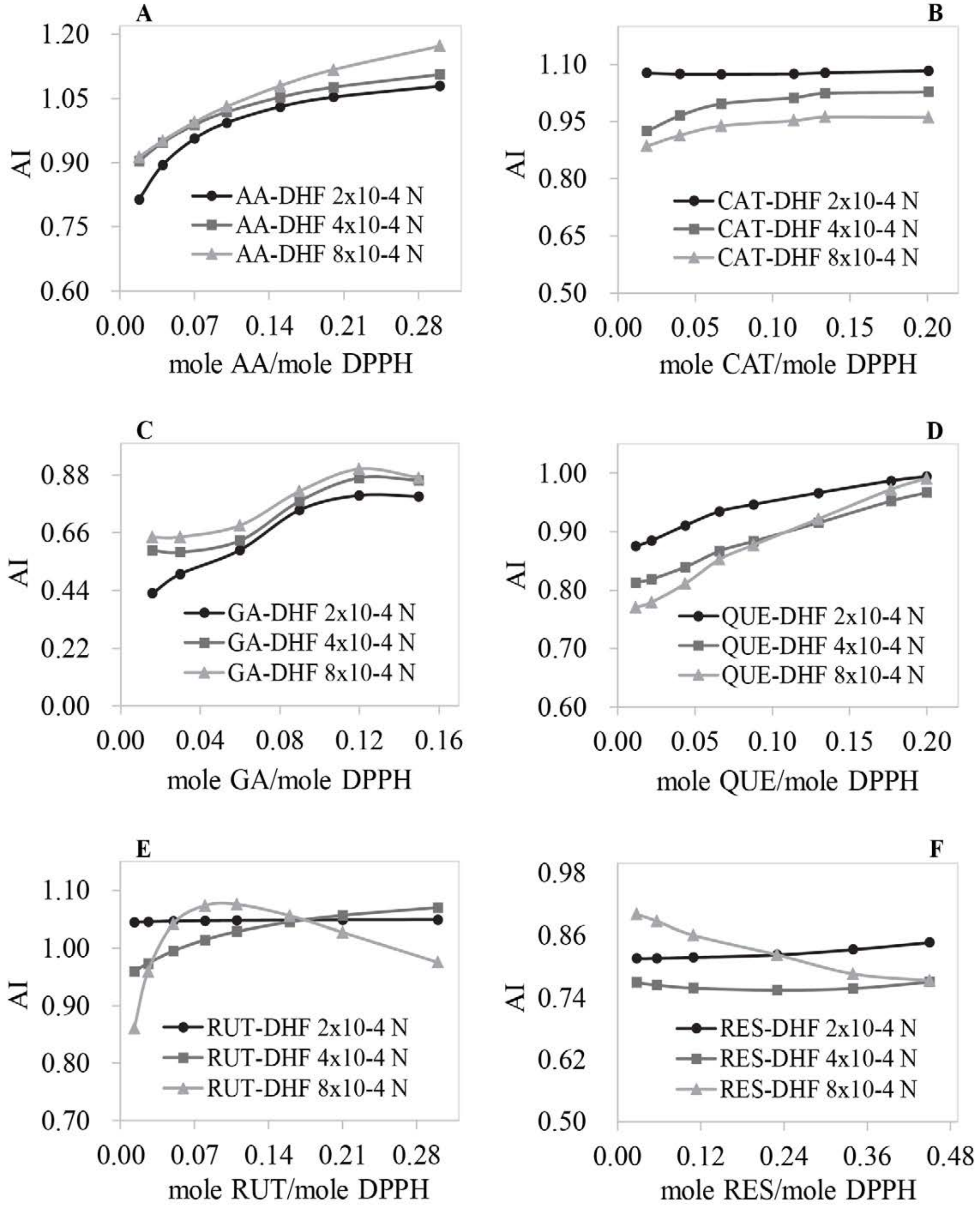

Figure 2. AI of AA and phenolic compounds in the combination with different concentrations of DHF. Data are presented as mean values (n ≥ 3).

that after 30 min of AA – DHF interaction with DPPH$^•$, dose – dependent synergistic effects are registered, confirming our suppositions.

The AI values of AA – DHF combinations evolve from antagonistic to synergistic ones as a consequence of the increase of both antioxidants' concentrations. The highest synergistic value of 1.17 was obtained for the mixture of 0.30 AA/DPPH molar ratio with 8×10^{-4} N of DHF (Figure 2A). By using NMR spectroscopy, it was made an attempt to understand the mechanism of synergistic antioxidant action of AA – DHF mixtures in the reaction with DPPH$^•$.[75] The hypothesis of a mutual regeneration of antioxidants proved to be valid only in deuterated methanol – chloroform solvents, where partial regeneration of the dehydroascorbic acid by the DHF was established.[75] Therefore, it was admitted that in 96% EtOH some regeneration processes can also occur. On the other hand, the regeneration by AA of the oxidized form of DHF has not been demonstrated. Moreover, the keto groups characteristic to the DHF oxidized form, that were expected to appear in the NMR spectra, have not been detected. Still, the redox reaction between DHF and DPPH$^•$ did occur as demonstrated by the change in colour of the radical from purple to dark yellow. Previously, it was reported that DHF decomposes spontaneously in aqueous solutions to form carbon dioxide and glycolaldehyde via two consecutive first – order reactions.[68,76] The highest rates of decarboxylation process are in the pH range of 2 – 3.5, because at low pH the keto – enol equilibrium is shifted to the keto form of DHF, which is instable and decomposes.[76–78] In our case, the solutions formed of 96% EtOH, AA and DHF possess relatively high acidity – 3.64 to 4.04 (Table 2), characterized by the prevailing of keto form,[77] that may determine partial decarboxylation of DHF.

The AI between CAT and DHF

The AI between CAT and DHF (Figure 2B) demonstrate good synergistic effects. The majority of synergistic AI's values (maximum of 1.08) can be noticed in the mixtures with the lowest concentration of DHF (2×10^{-4} N). Samples containing 4×10^{-4} N and 8×10^{-4} N of DHF demonstrated mostly additive AI, this indicating that the increase of the DHF content is disadvantageous for the total antioxidant activity of the mixtures. The reason behind this effect may be the lowering of the pH caused by higher concentrations of DHF, which negatively affects CAT antioxidant activity. Several authors also reported synergistic interactions between strong antioxidants and phenolic compounds,[12,15,21,74] and suggested as explanation the regeneration mechanism of action. The DHF is a strong and fast antioxidant[6,27] and it will be the first to interact with DPPH$^•$, before CAT, thus, the regeneration of CAT by DHF can be excluded. Also, the decarboxylation of oxidized form of DHF in the acidic solution makes impossible its reduction to the initial form by the CAT.

In this case, the operative mechanism of action appears to be the formation of adducts and oligomeric compounds. Similar dose – dependent synergistic behavior of CAT was noticed in combination with AA.[15] Different molar ratios of CAT/AA demonstrated both enhanced antioxidant activity and prooxidant effect.[15] Authors[15,44,46] found the oligomerization of CAT with subsequent formation of procyanidin structures to be a decisive factor influencing the antioxidant – prooxidant balance, and, therefore, the type of AI.

The AI between GA and DHF

The GA – DHF mixtures show mainly antagonistic effects ranging from 0.43 to 0.82. From Figure 2C it can be noticed that the addition of larger concentrations of DHF slightly improves the total antioxidant activity, however, the majority of the interactions remain in the range of antagonistic values. Unlike the AA – DHF and QUE – DHF (Figure 2D) interactions which follow only antioxidant ascending tendencies, the example of GA – DHF does not respect it. The highest AI value of 0.90 can be observed for 0.12 GA/DPPH molar ratio in combination with 8×10^{-4} N of DHF

Based on the data reported by Piang – Siong et al.[9] on the GA's AI with *trans*-aconitic acid, which have similar structural units as DHF, comparable synergistic effects have been expected from GA – DHF interactions. On the other hand, GA in combination with different concentrations of AA showed strong antagonistic effects.[79] Other antagonistic effects of the mixtures of phenolic compounds and GA have been recently described.[80,81] Authors suggested that the antagonism is a consequence of the weaker antioxidant regeneration by the strongest one – hypothesis that was supported by the analysis of the reduction potentials.[81] Also, the effect of the difference in reaction kinetics between the antioxidant and free radical has been implied.[80]

The oligomerization of GA was also admitted, however, according to the existing data, the polymerization of GA would enhance the antioxidant activity of the reaction products,[82,83] which is in discrepancy with our results.

The AI between QUE and DHF

The interaction of QUE with DHF shows mainly additive effects which did not exceed the 0.99 value. According to Figure 2D, larger concentrations of DHF affects negatively the total antioxidant activity of the mixtures, and produce antagonistic effects. These outcomes were unforeseen since in the literature are described synergistic regenerative interactions between QUE and AA, AA being a strong antioxidant like DHF.[12,21] Other authors[84,85] also found synergistic interactions of the mixture QUE – AA, and of QUE with other class of compounds.[86,87] The additive effects found in the experiment can be due to the DHF decarboxylation, which would make impossible the reduction of *o*-quinone to QUE, and by this, excludes the hypothesis of mutual regeneration of QUE and DHF.

The AI between RUT and DHF

Good antioxidant activity is revealed in the mixtures of RUT and DHF, with a preponderance of synergistic interactions in the range of 1.05 – 1.08 (Figure 2E). The concentration of DHF affects the total antioxidant activity and the AI. The mixtures consisting of different concentrations of RUT and 2×10^{-4} N of DHF possess synergistic effects of 1.05 independently of the change of RUT's content. For the second concentration of DHF – 4×10^{-4} N, the AI start at the value 0.96 and increases till 1.07 once the RUT/DPPH molar ratio is augmented. At 8×10^{-4} N of DHF, the highest synergistic effect of 1.07 can be noticed for the 0.11 RUT/DPPH moral ratio, followed by a significant decrease of the AI.

Similar synergistic results have been obtained by Tavadyan et al.[88] in binary mixtures of bioflavonoids and AA or trolox, using the ORAC method. They observed synergistic interactions of AA with flavonoids RUT and naringin that have O-glucosyl group in the molecular structure; in the same time, AA in combination with QUE and morin showed antagonistic effect. These findings are in accordance with our data on the QUE and RUT antioxidant interaction with DHF (Figure 2, cases D and E). Tavadyan et al.[88] suggested that the enhanced antioxidant activity of RUT – AA mixture is due to the presence of the glycoside in its structure that condition the formation of intramolecular hydrogen bonds between hydrogen atoms and phenolic OH groups responsible for the interaction with radicals and two-electron-donor oxygen atoms of the glucosyl substituent. In the situation of RUT – DHF interactions, the O-glucosyl group can influence positively the stability of DHF, and prevents the decarboxylation process. This idea is supported by recent revelations on the polymerization process involving the DHF, acetone and methanol.[89] Therefore, such situation would enable some regeneration processes between RUT and DHF.

The AI between RES and DHF

The combination of RES and DHF showed mostly antagonistic effects (0.75 – 0.83) and only few additive interactions (0.85 – 0.90) (Figure 2F). Samples containing RES and the first two concentration of DHF – 2×10^{-4} N and 4×10^{-4} N, demonstrate that the increase in the content of organic acid lead to drastic decrease of AI values. For mixtures with 8×10^{-4} N DHF the AI evolve negatively – from additive to antagonistic effects, with the increase of the RES content.

Other investigations reported both synergistic and antagonistic interactions between RES and various phenolic compounds, with the prevalence of the antagonistic ones.[11,13,81] In reactions with free radicals, RES yields various oligomers as final products.[54,90,91] The NMR analysis showed that in combination with AA, the oxidation of RES generates viniferins.[92] In this case, the regeneration mechanism of synergistic antioxidant action can be excluded. We assume that in the presence of DHF, the polymerization of RES could be accelerated, this would reduce the number of OH group available for free radicals scavenging, like it was the case of other phenolic compounds.[15,39]

4. Conclusions

Organic acids affect the antioxidant activity of phenolic and non-phenolic natural compounds. Their presence can lead to synergistic, additive or antagonistic interactions depending on the antioxidant/organic acid, antioxidant/free radical molar ratio, acidity, mechanism of action, etc. The TA and CA significantly affect the antioxidant activity of CAT, GA, QUE and RES, their combinations mainly causing additive and antagonistic interactions. This can be justified by the fact that phenolic compounds are less susceptible to oxidation and polymerization at high pH. Such behavior is favorable in multicomponent phenolic mixtures, where polymerization followed by a decrease in electron donating groups has been observed. Organic acids in combination with RUT enhance, the antioxidant activity of polyphenol, showing strong synergistic effects. We assume that some polymerization processes took place and the final products have higher antioxidant activity against DPPH$^\bullet$. The combination of AA and TA or CA shows good synergistic interactions, which may be due to the suppression of the SPLET mechanism of AA antioxidant activity and the promotion of the HAT mechanism.

The DHF added to phenolic and non-phenolic compounds demonstrates dose–dependent AI, which may be related to the high antioxidant activity of the organic acid. The combinations of CAT or RUT with DHF show good synergistic effects along with additive effects; with QUE, additive and antagonistic AI are observed. The hypothesis of mutual regeneration of polyphenols and DHF is generally discarded since the decarboxylation of DHF in acidic solutions has already been demonstrated. An exception is the combination RUT–DHF, where the O-glucosyl group of RUT may positively affect the stability of DHF and prevent the decarboxylation process. Higher concentrations of AA in combination with DHF show good synergistic effects against DPPH$^\bullet$, but also additive and antagonistic effects at lower AA and DHF concentrations. The GA and RES mixed with DHF show mainly antagonistic effects. It is suggested that the antagonistic AI is the consequence of the decarboxylation of DHF, together with adverse polymerization processes and the effect of the different reaction kinetics between the antioxidant and DPPH. Further studies and experimental data are needed to confirm these conclusions and to clarify the mechanisms of antioxidant action.

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Povzetek

Antioksidativne interakcije med več naravnimi fenolnimi in nefenolnimi spojinami (katehin, kvercetin, rutin, resveratrol, galna kislina in askorbinska kislina) ter organskimi kislinami (vinska, citronska in dihidroksifumarna kislina) smo proučevali z metodo 2,2-difenil-1-pikrilhidrazil (DPPH). Pri kombinacijah katehina, kvercetina, resveratrola in galne kisline z vinsko in citronsko kislino so bile ugotovljene glavne aditivne in antagonistične interakcije; tako vedenje je lahko posledica večje stabilnosti fenolnih spojin v kislih medijih. Rutin in askorbinska kislina sta pokazala dobre sinergijske učinke z vinsko in citronsko organsko kislino, kar je lahko posledica polimerizacijskih procesov pri rutinu in spremembe mehanizma delovanja pri askorbinski kislini. Mešanice v kombinaciji z dihidroksifumarno kislino so pokazale od odmerka odvisne sinergijske, aditivne ali antagonistične antioksidativne interakcije. Dobri sinergijski učinki so bili opaženi pri binarnih mešanicah dihidroksifumarne kisline z askorbinsko kislino, katehinom in rutinom.