ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Flavonoid-Ascorbate Mixtures Ratio - Antioxidant Activity Relationships.

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ABSTRACT

Component ratio – antioxidant activity relationships were evaluated for binary mixtures of natural bioflavonoid product Diquertin and a number of flavonoids (dihydroquercetin, naringenin, quercetin, rutin) with ascorbic acid or α-tocoferol. Antioxidant activity was measured employing the Trolox Equivalent Antioxidant Capacity assay. Component ratio – antioxidant activity relationships were found to differ for various flavonoid and ascorbic acid combinations. Diquertin+ascorbic acid and Dihydroquercetin+ascorbic acid combinations showed antagonism, which was augmented with increasing ascorbic acid content in the mixture. Naringenin+ascorbic acid combinations demonstrated synergy in cases of naringenin prevalence and antagonism in cases of ascorbic acid prevalence. Quercetin+ascorbic acid and rutin+ascorbic acid combinations showed both antagonism and additive effects, whereby antagonism intensified with increasing flavonol content in binary mixtures. α-Tocopherol+ascorbic acid and α-tocopherol+Diquertin combinations showed insignificant antagonism, which did not depend on the component ratio.

Keywords: flavonoid; diquertin; dihydroquercetin; ascorbic acid; antioxidant activity;

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ISSN: 0975-8585

INTRODUCTION

Flavonoids and ascorbic acids (AA) are common components of herbal food sources that exhibit a wide variety of biological activities. Nevertheless the effects of their co-presence on each other's biological activity, including antioxidant activity (AOA), is not completely understood. An important factor to consider is possible interference between food/ beverage antioxidants and antioxidants taken as food supplements and/or therapeutic products. Though, as noted by A. Szent-Györgyi and colleagues,[1–3] flavonoids can play an ascorbate-protective role, the study of their interaction still leaves some questions. *In vitro* studies have demonstrated that AA, in its turn, can play a flavonoid-protective role. In particular, the addition of AA to a stabilized reaction system with an almost completely depleted reduced form of a flavonoid (i.e. AA addition after flavonoid addition) resulted in more profound inhibition of $DPPH^{\bullet}$ radical level as compared to adding AA simultaneously or before flavonoid addition[4]. This beneficial effect may have been achieved due to AA interaction with oxidized forms of the flavonoid; AA has been shown to prevent oxidative degradation of anthocyanidines,[5] quercetin[6] and catechin.[7]. Bors and colleagues have reported that flavonoids with a double bond C2-C3 in the C-ring and an ortho-dihydroxy substitution in the B-ring (pyrocatechol type) have a higher redox-potential than ascorbate [8] and are therefore not able to play an ascorbate-protective role; only dihydroquercetin could reduce the ascorbyl radical.

Diquertin (DKV) is a natural bioflavonoid product, isolated from the wood of the *Larix sibirica* Ledeb. or *Larix gmelinii* Rupr, which contains not less than 90% of dihydroquercetin (Tjukavkina et al.. 1997). Minor components of DKV are naringenin, dihydrokaempferol and quercetin. DKV and its main component dihydroquercetin show a wide range of biological and pharmacological activities [9–12]. Of our particular interest is the study of the DKV+AA combinations which improve blood rheology[13,14], demonstrate cerebroprotective effects [15]. DKV and its constituents were chosen as a base components of mixtures "Flavonoid+AA". All combinations were studied in a wide range of component ratios in order to reveal tendencies of the component ratio's effect on the AOA.

MATERIALS AND METHODS

Chemicals

DKV (Flavir, Russia) was characterized by HPLC-UV as follows: dihydroquercetin 90,23±0,32%; dihydrokaempferol 0,67±0,05%; naringenin 0,28±0,03%; quercetin 2,21±0,11%. Dihydroquercetin (primary pharmaceutical reference standard in Russia, purity > 99%, Flavir, Russia). Quercetin, naringenin and rutin (Acros Organics, USA); AA, phosphate-buffered saline (PBS, pH 7,4) and trolox (Sigma, USA); α- tocopherol (Fluka, Switzerland), ABTS [2,2/-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid) diammoniuim salt] (Tokio Chemical Industry, Japan); potassium persulfate and sodium chloride (Sigma-Aldrich, USA). All other chemicals used were of analytical grade and prepared on the day of use.

AOA measurement

We employed TEAC assay for measurement of combination AOA [16]. All experiments were performed at least in triplicate. Student's t-test was used for comparison of means. A difference was considered statistically significant at $P < 0,01$.

TEACg was calculated as a ratio of slopes of the studied substances to Trolox. $TEAC_M$ value was re-calculated as follows: $TEAC_M$=TEACg×M/1000, where M is the molar mass of the studied substance.

Mixture effect (ME) calculations

Mixture effect was calculated according to equation: ME= EI/TI, где EI – experimental ABTS•+ inhibition, %, and TI – theoretical ABTS•+ inhibition, %. TI was calculated from linear regression equations for each compound of a binary mixture in appropriate concentrations as follows: TI=inh%1+inh%2=(k1x1+b1)+(k2x2+b2), where x1 and x2 – concentrations of antioxidant components of a binary mixture, mg/L, k1 and k2 – slopes, and b1 and b2 – intercepts for components of binary mixture measured alone. ME>1 corresponds to synergy, ME=1 to additive effect, and ME<1 to antagonism.

ISSN: 0975-8585

RESULTS AND DISCUSSION

Antioxidant activity of individual compounds

Data for individual substances are presented in Table 1. The studied antioxidants can be ranked by $TEAC_M$ values as follows: quercetin > rutin > DKV ≈ dihydroquercetin > naringenin > α-tocopherol ≈ AA ≈ Trolox. In general, the AOA descending order resulting from this study corresponds well to previously obtained data [16]. DKV and dihydroquercetin show virtually the same AOA (the difference is statistically non-significant), despite the presence of other minor flavonoids in DKV along with dihydroquercetin (including quercetin, which exhibits higher AOA). These components seem to provide an insufficient contribution to the total AOA of DKV.

Table 1: Calibration curve parameters, TEAC values and concentration ranges of antioxidants and $ABTS^{\bullet+}$ in the reaction mixture.

	k±SD			b±SD			r	TEACg	$TEAC_M$	concentration range, μM			$C_{ABTS\bullet+}$ μM
DKV	40.5	±	0.6	4.2	±	0.5	0.985	9.47	-	0.3	–	6.3[a]	43-50 μM
Dihydroquercetin	38.6	±	0.8	2.3	±	0.4	0.996	9.01	2.74	0.4	–	4.2	
Quercetin	88.8	±	2.3	2.4	±	0.6	0.990	20.73	6.27	0.2	–	1.5	
Rutin	24.4	±	0.5	2.5	±	0.4	0.994	5.69	3.47	0.3	–	2.6	
Naringenin	37.8	±	0.9	2.9	±	0.8	0.992	8.82	2.40	0.3	–	7.6	
α-Tocopherol	9.1	±	0.1	1.8	±	0.2	0.999	2.12	0.91	0.7	–	10.9	
Ascorbic acid	21.7	±	0.3	1.8	±	0.5	0.995	5.07	0.89	0.7	–	13.8	
Trolox	4.3	±	0.1	1.5	±	0.7	0.998	-	1.00	4.0	–	10.2	

Note: [a]concentration range for DKV is given in terms of dihydroquercetin content
Calibration curves 'inhibition percentage – concentration' described by equation y=kx+b, where y the percentage of $ABTS^{\bullet+}$ inhibition, %, x the antioxidant concentration, mg/L, k the slope, and b the intercept.

Antioxidant activity of mixtures

Binary mixtures were prepared with component molar concentration ratios mainly varying from 10:1 to 1:10. Results of mixture effect determinations are presented in Table 2 and Figure 1.

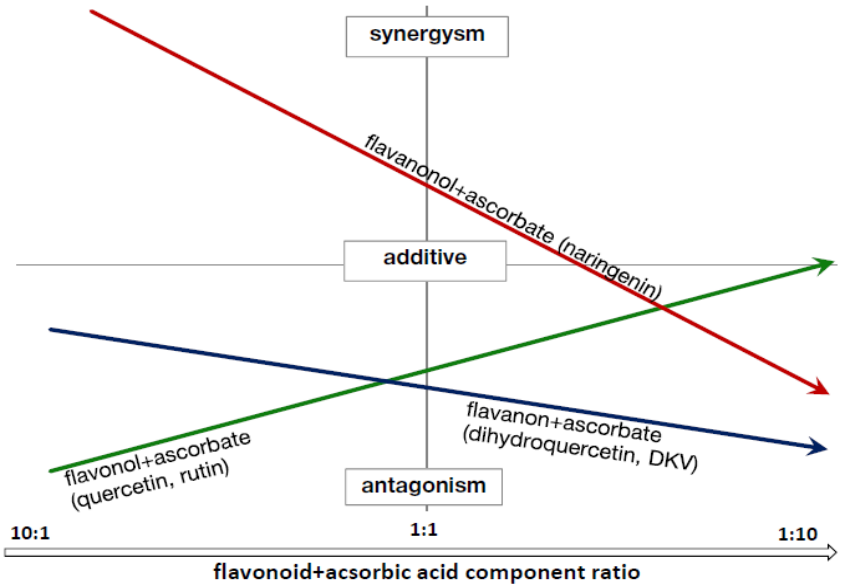

Figure 1: Component ratio – mixture effect (ME) relationships in flavoniod+ascorbic acid binary mixtures.

ISSN: 0975-8585

Table 2: Mixture effect of binary mixtures

mixture	N	ME	±	SD			mixture	n	ME	±	SD	
Dihydroquercetin+AA							*DKV[a]+AA*					
10:1	8	0.96	±	0.02	[b]		10:1	9	0.96	±	0.03	[b]
5:1	8	0.94	±	0.03	[b]		5:1	8	0.93	±	0.02	[b]
3:1	9	0.91	±	0.03	[b]		3:1	9	0.90	±	0.03	[b]
1:1	9	0.93	±	0.04	[b]		1:1	8	0.92	±	0.01	[b]
1:3	8	0.87	±	0.02	[b]		1:3	8	0.87	±	0.02	[b]
1:5	8	0.89	±	0.02	[b]		1:5	8	0.89	±	0.03	[b]
1:10	8	0.84	±	0.01	[b]		1:10	8	0.87	±	0.01	[a]
Quercetin+AA							*Rutin+AA*					
10:1	8	0.87	±	0.02	[b]		2.8:1	12	0.83	±	0.03	[b]
5:1	8	0.87	±	0.02	[b]		1.4:1	8	0.92	±	0.03	[b]
3:1	8	0.85	±	0.01	[b]		1:1.2	11	0.92	±	0.03	[b]
1:1	8	0.97	±	0.01	[b]		1:3.5	9	0.97	±	0.04	
1:3	8	0.96	±	0.01	[b]		1:10	8	0.96	±	0.05	
1:5	8	0.96	±	0.02	[b]		1:18	10	1.04	±	0.03	[b]
1:10	9	0.95	±	0.02	[b]							
Naringenin+AA							*DKV[a]+α-Tocopherol*					
10:1	10	1.21	±	0.05	[b]		5:1	8	0.90	±	0.04	[b]
6.3:1	8	1.14	±	0.04	[b]		2:1	8	0.90	±	0.07	[b]
5:1	8	1.14	±	0.03	[b]		1:1	8	0.91	±	0.03	[b]
3.2:1	12	1.18	±	0.05	[b]		1:2.5	8	0.94	±	0.05	[b]
3:1	8	1.14	±	0.04	[b]		1:5	8	0.91	±	0.03	[b]
1.9:1	12	1.17	±	0.08	[b]							
1:1	9	1.04	±	0.03	[b]		*α-Tocopherol+AA*					
1:1.6	9	1.05	±	0.07			5:1	8	0.94	±	0.02	[b]
1:3	9	0.98	±	0.03			1:1	8	0.94	±	0.02	[b]
1:4.7	12	0.95	±	0.04	[b]		1:5	8	0.93	±	0.02	[b]
1:5	8	0.94	±	0.03	[b]							
1:7.9	11	0.92	±	0.04	[b]							
1:10	9	0.91	±	0.04	[b]							
1:16	8	0.94	±	0.03	[b]							

Note: [a]molar ratio in terms of dihydroquercetin content
[b]statistically significant, two-sample t-test, p=0,01

DKV+AA and Dihydroquercetin+AA

All combinations showed antagonism, which was augmented with increasing AA content. The antagonistic effect of the DKV+AA combination was virtually the same as for the dihydroquercetin+AA. Maximum antagonism (ME=0.84±0.01) was found at dihydroquercetin+AA 1:10 ratio.

Quercetin+AA and rutin+AA

Quercetin+AA and rutin+AA combinations also showed antagonism, but in contrast to dihydroquercetin-containing combinations, minimal ME (0.83±0.03) was found for flavonoid, but not AA prevalence (3:1 for quercetin+AA and 2.8:1 for rutin+AA). The observed antagonism attenuated or became statistically non-significant when the component ratio was changed to equal or AA prevalence.

Naringenin+AA.

The naringenin+AA combination was the only binary mixture that showed synergy of the components. This effect was found in all cases of naringenin prevalence in the combination, with the greatest effect at 1.21±0.05 at a 10:1 ratio. Component ratios 1:1.6 - 1:3 showed additive effects, and a further increase of AA content resulted in antagonism with minimum ME 0.91±0.04 at a 1:10 ratio.

DKV+α-tocopherol and α-tocopherol+AA

All DKV+α-tocopherol and α-tocopherol+AA combinations demonstrated slight antagonism. However, in contrast to flavonoid+AA combinations, the effect did not depend on the component ratio.

Structure – ME relationships

In general, antagonism, albeit slight, was found for the majority of combinations. Taking into account an assumption that antagonism results from an utilization of a stronger antioxidant to regenerate the weaker one (and vice versa in the case of synergy), ME appears to be determined by the following interactions: Flavonoid interaction with $ABTS^{\bullet+}$ leading to oxidized forms of the flavonoid (Fl_{ox}, Figure 2, route 1), AA interaction with $ABTS^{\bullet+}$ leading to oxidized forms of AA (A_{ox}, Figure 2, route 2), Flavonoid interaction with AA_{ox} (AA regeneration, ascorbat-protective role, Figure 2, route 3), AA interaction with Fl_{ox} (flavonoid regeneration, flavonoid-protective role, Figure 2, route 4). Structural differences may explain the different means of stabilization among flavoxyl radicals, including interaction with AA and/or its oxidized derivatives.

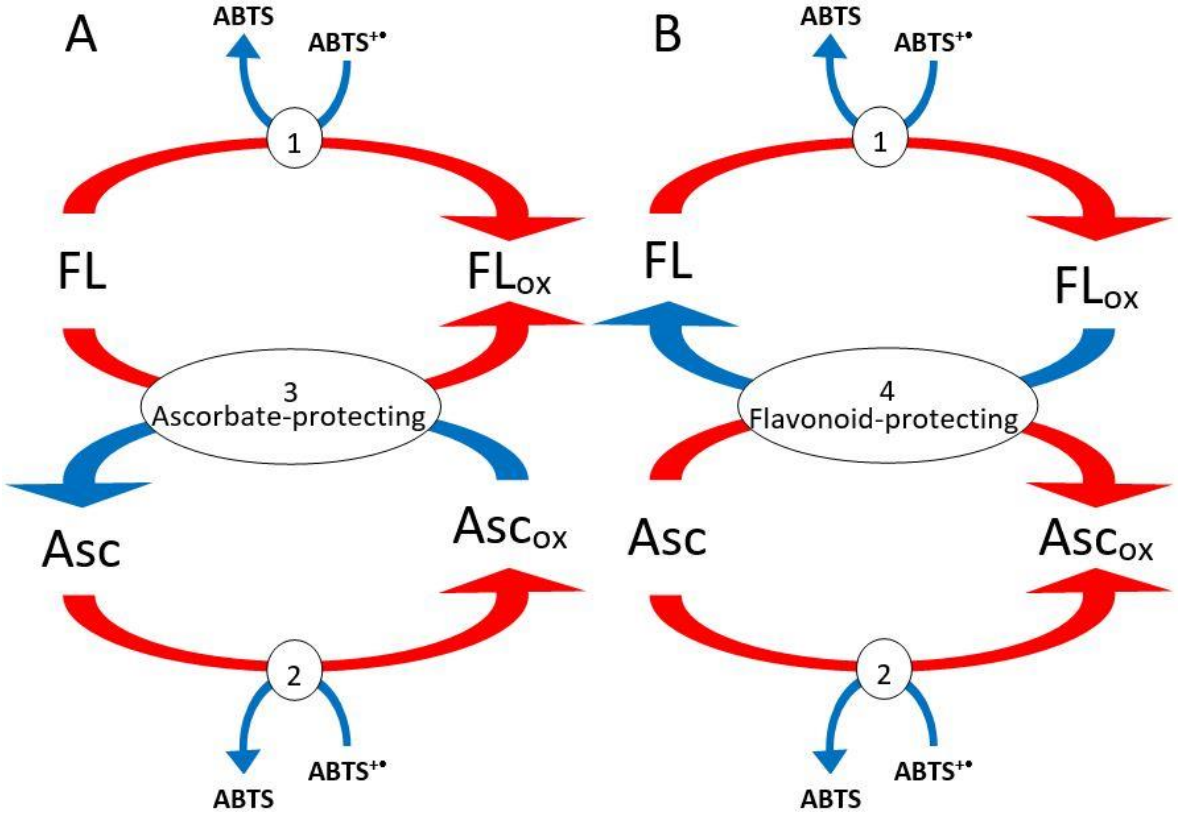

Figure 2: Possible routes of components interaction in studied flavonoid+ascorbic acid binary mixtures.
Note: (1) Flavonoid (FL) interaction with $ABTS^{\bullet+}$ leading to oxidized forms of the flavonoid (Fl_{ox}); (2) ascorbic acid (AA) interaction with $ABTS^{\bullet+}$ leading to oxidized forms of AA (A_{ox}); (3) Flavonoid interaction with AA_{ox} (AA regeneration, ascorbat-protective role); (4) AA interaction with Fl_{ox} (flavonoid regeneration, flavonoid-protective role)

An antagonistic interaction between quercetin, rutin, dihydroquercetin and AA can result from route 3; therefore, these flavonoids play an ascorbate-protective role, which lead to their depletion and, subsequently to a decreased total AOA of the combination (due to the lower AOA of regenerated AA as compared to flavonoids).

The absence of synergy in the above mentioned combinations indicates that AA does not regenerate flavonoids by route 4.

The quercetin+AA and rutin+AA mixture effects, which are close to additive in cases of AA prevalence, supposes the domination of the route 1 over routes 2 and 3. Quercetin and rutin are characterized by a common electron distribution over all rings due to overlapping of π-orbitals of all atoms in the flavonol molecule; therefore, they have a planar structure (which was confirmed for quercetin by X-ray structural analysis[17]). The common electron distribution over the flavonol molecule probably assures the high stability of the resulting flavoxyl radical, as well as, apparently, the rapid reduction of $ABTS^{\bullet+}$ radical-cation present in the system. Antagonism was observed for combinations with flavonol prevalence; in these cases, there is the apparent possibility of flavonol interactions, both by major route 1 and by route 3 with oxidized AA forms.

There is no common electron distribution over the dihydroquercetin molecule, but there is a possibility of stabilization of the bi-radical product of di-electronic oxidation by the formation of a B-ring quinoid structure. Due to a slower dihydroquercetin interaction with $ABTS^{\bullet+}$ as compared to AA, ME is probably determined by AA_{ox} concentration and interaction with the flavonoid by route 3. This explains antagonism growth with increasing AA content.

Naringenin+AA mixtures demonstrate both antagonism and synergy in contrast to quercetin, rutin and dihydroquercetin mixtures with AA, thus an additional interaction by route 4 can be assumed. Naringenin lacks the C2-C3 double bond and B-ring pyrocatechine fragment, and it seems to be the reason of the relatively slow naringenin interaction with $ABTS^{\bullet+}$. In cases of AA prevalence, routes 1 and 3 compete with each other, thus leading to antagonism. Development of synergy upon elevation of naringenin content suggests similar naringenin and AA redox-potentials, and can be explained by competition of routes 2 and 4.

TEAC assay effect on ME

One of the major factors that determine ME is the model system used to measure the AOA. Our understanding of the process model used in the employed TEAC assay is provided below. Upon the addition of an antioxidant to the reaction mixture, the latter will contain $ABTS^{\bullet+}$ radical cation, antioxidant and the reduced and intact ABTS. $ABTS^{\bullet+}$ radical cation basal concentration in the mixture (43 – 50 μM) is approximately 10-20 higher than that of antioxidant (Table 1). Thus there is an excess of $ABTS^{\bullet+}$ radical cation during the whole incubation period, which stimulates the maximal possible AOA of the antioxidant. If intermediate products formed in the mixture have their own AOA, they can also interact with $ABTS^{\bullet+}$. As a result, the equilibrium of the reaction $ABTS^{\bullet+} + AO \rightarrow ABTS + AO^{\bullet}$ is supposed to be shifted to the right as much as possible. Another logical consequence of $ABTS^{\bullet+}$ excess will be the higher probability of antioxidant interaction with $ABTS^{\bullet+}$ as compared to interaction with an oxidized form of another antioxidant, and reduction of the latter leading to any ME. As a result, antagonistic or synergystic effects are respectively small (ME not exceeding 1±0.2 range). Our conclusions are also supported by studies using a similar model of $ABTS^{\bullet+}$ radical cation inhibition, whereby combinations of chlorogenic acids with polyphenols demonstrated slight synergy (up to 8.3%) or antagonism (up to 5.5%).[18] The TEAC assay method (ABTS/PP end-point model) used in this study can be considered as a model for the investigation of conditions in which radical concentrations significantly exceed those of antioxidants.

On the one hand, the above suggestions limit the applicability of TEAC assay for ME measurement, while on the other, knowledge of combination composition allows for accurate prognosis of its TEAC based on component AOA data, thus a comparison of theoretical, product composition-based TEAC with the actually measured TEAC may indicate the presence of other, unknown antioxidants.

CONCLUSIONS

Our study demonstrated that the ME of flavonoid+AA combinations depended on the component ratio. For flavonol (quercetin or rutin) + AA combinations, the increase of flavonol content resulted in a trend of ME change from an additive effect to antagonism combinations with AA. An opposite tendency was found for flavanonol dihydroquercetin, flavanone naringenin and bioflavonoid product Diquertin, whereby increases of their content was accompanied by ME change from antagonism to an additive effect (for dihydroquercetin and Diquertin), or even to synergy (for naringenin).

ISSN: 0975-8585

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