Original Contribution

PROTECTIVE EFFECTS OF NATURAL BIO-ANTIOXIDANTS AND THEIR SYNTHETIC ANALOGUES IN EQUIMOLAR BINARY AND TRIPLE MIXTURES

V. D. Kancheva*

Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT

PURPOSE: Different effects (synergism, additivism or antagonism) of binary and triple mixtures of bio-antioxidants with alpha-tocopherol (TOH) and/or ascorbyl palmitate (AscPH) was studied. **METHODS:** Kinetics of bulk lipid autoxidation at 80°C was studied in absence and in presence of equimolar binary mixtures (1:1) with TOH or AscPH and of equimolar triple mixtures (1:1:1) of bio-antioxidants with TOH and AscPH at concentration of the individual components 0.1mM. Different effects was explain on the basis of the following formulae: A) For binary mixtures: Synergism: $IP_{1+2} > IP_1 + IP_2$; Additivism: $IP_{1+2} = IP_1 + IP_2$; and Antagonism: $IP_{1+2} < IP_1 + IP_2$; B) For triple mixtures: Synergism: $IP_{\Sigma} > \Sigma IP_i$; Additivism $IP_{\Sigma} = \Sigma IP_i$; and Antagonism: $IP_{\Sigma} < \Sigma IP_i$, where $i = 1, 2, 3$ components. TOH content in the studied mixtures at different reaction time of lipid autoxidation was monitored by using HPLC method. **RESULTS:** Mechanisms of action of bio-antioxidants in binary and triple mixtures is presented. It has been proven that the synergism obtained between binary and triple mixtures is due to the significant effect of TOH regeneration. **CONCLUSIONS:** The practical application of results obtained is discussed.

Key words: protective effects, antioxidant mixtures, bio-antioxidants, HPLC.

INTRODUCTION

Biologically active compounds with antioxidant potential, i.e. bio-antioxidants (natural and their synthetic analogues) have a wide range of applications. They are important drugs, antibiotics, agrochemical substitutes, and food preservatives. Many of the drugs today are synthetic modifications of naturally obtained substances with both activities-biological and antioxidant. The most important known natural bio-antioxidants are flavonoids and phenolic acids. Last decades the attention of scientists was focused on design of new bio-antioxidants. The idea is to combine in one molecule by a specific synthetic way various functional fragments, responsible for biological and antioxidant activities for the individual compounds. When it

is not possible it can be used so called bio-antioxidant compositions – by mixtures (double or triple) of biologically active compounds, antioxidants, synergists, which are multifunctional (1-3).

Nowadays bio-antioxidants play an important role in prevention of human diseases as components of food additives and for treatment of different diseases as monotherapy or in complex therapy with drugs. It has been found that in the first stage of the development of atherosclerosis the system works in its normal regime. The introduction of antioxidants in the affected body normalizes not only the peroxide oxidation, but also the lipid content. Monotherapy with antioxidants also is used in early stage of atherosclerosis and in oncology. In this case the antioxidants are used at high concentration. There are a lot of reports about combination therapy with drugs and antioxidants, but in this case antioxidants are mainly used as additives in the complex tumor therapy - the antioxidant is in low

*Correspondence to: Assoc. Prof. Dr. Vessela D. Kancheva, Lipid Chemistry Department, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. "Georgi Bonchev" Str., Bl. 9, 1113 Sofia; Phone: 02-9606-187; Fax: 02-8700-225; e-mail: vedeka@abv.bg

concentrations. In this respect the medical treatment of most of diseases includes formulations based on a combination of traditional drugs with targeted functionality and different antioxidants (2, 4).

For that reason, it is of importance to be studied different effects of equimolar binary and triple mixtures of some known and new bio-antioxidants with α -tocopherol (TOH) and ascorbyl palmitate (AscPH). The aim of this study is to check which antioxidant compositions are able to ensure the highest lipid oxidation stability and different effects (synergism, additivism or antagonism) of binary and triple mixtures of bio-antioxidants with α -tocopherol (TOH) and/or ascorbyl palmitate (AscPH).

MATERIAL AND METHODS

DL- α -tocopherol (TOH), ferulic acid (FA), caffeic acid (CA), sinapic acid (SA), butylated hydroxytoluene (BHT), tret-butylhydroquinone (TBHQ) and ascorbyl palmitate (AscPH) were from E. Merck (Germany). Quercetin (Qu), luteolin (Lu) and rutin (Ru) were isolated earlier from *Carthamus lanatus* L. (5). 7,8-dihydroxy-5-methyl-coumarin (Cum₁), 6,7-dihydroxy-4-methyl-coumarin (Cum₂) and 7-Hydroxy-4-methyl-coumarin (Cum₃) were synthesized from University of Delhi, India and previously reported (6). Dehydrozingerone (M₁OH), zingerone (M₂OH), dimer of dehydrozingerone D₁(OH)₂, dimer of zingerone D₂(OH)₂ and dimer of ferulic acid (DFA) was synthesized from CNR Institute of biomolecular chemistry, Italy and reported previously (7). A Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan), consisting of a LC-10AD pump, SCTL 10A system controller and SPD-M 10A photodiode array detector was employed. A detailed protocol can be seen in Ref. (8)

Lipid samples. Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and anti-oxidants by adsorption chromatography and stored under nitrogen at minus 20°C. Lipid samples containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone were added to the lipid sample. Solvents were removed under a nitrogen flow.

Lipid autoxidation. The process was carried out in a thermostatic bath at 80 °C ($\pm$ 0.2 °C) by blowing air through the samples in special vessels. The oxidation process was monitored by

withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary products (lipid hydroperoxides, LOOH) concentration, i.e. the peroxide value (PV) (9). All kinetic data are expressed as the average of two independent measurements which were processed using the computer programs Origin 6.1 and Microsoft Excel-97.

Synergism, additivism, antagonism. If two or more antioxidants are added to oxidizing substrates, their combined inhibitory effect can be synergistic, additive, or antagonistic (9,10).

Synergism – the inhibiting effect of the binary and triple mixtures (IP_Σ) is higher than the sum of the induction periods of the individual phenolic antioxidants (IP_i), i.e. IP_Σ > ΣIP_i, where i = 1, 2, 3. **Additivism** - the inhibiting effect of the binary and triple mixtures (IP_Σ) is equal to the sum of the induction periods of the individual phenolic antioxidants, i.e. IP_Σ = ΣIP_i. **Antagonism** - the inhibiting effect of the (IP_Σ) is lower than the sum of the induction periods of the individual phenolic antioxidants, i.e. IP_Σ < ΣIP_i. The percent of the synergism was calculated according to the formulae created from Frankel (4) for the binary mixtures and updated for triple mixtures by us: % Synergism = 100 [(IP_Σ - ΣIP_i) / ΣIP_i]. We also suggest for the first time by the similar way the calculation of % antagonism by the formulae: % Antagonism = 100 [(ΣIP_i - IP_Σ) / IP_Σ].

Statistical analysis of IP determination. The standard deviation (SD) for different mean values of IP was (in h): IP=2.0, SD=0.2; IP=5.0, SD=0.3; IP=15.0, SD=1.0; IP=25, SD=1.5; IP=50.0, SD=3.0. The SD of PV determination (in meq/kg), according to the modified iodometric method for different mean values of PV, was: PV=12.0, SD=1.0; PV=30.0, SD=2.0; PV=70.0, SD=5.0; PV=150.0, SD=10; PV=250.0, SD=20. The R_A and R_C were quite constant varying by less than 2%.

RESULTS AND DISCUSSION

Protective effects of binary mixtures of flavonoids

Flavonoids are the well-known natural bio-antioxidants, because they are powerful antioxidants and possess various biological activities. A lot of studies of scientists are focused on the structure-activity relationship of these interesting compounds.

Figure 1. Structures of flavonoids: quercetine (Qu), luteoline (Lu) and rutine (Ru)

Figure 1 presents the structures of selected flavonoids under study. In order to check the possible synergism between two flavonoid aglicones Qu and Lu binary mixtures with equimolar concentrations (0.1mM) were prepared and their effect on the kinetics of lipid autoxidation was studied (**Figure 2 and Table 1**). The mixture of Qu+Lu showed almost 7-fold higher inhibiting effect than Lu and 1.4-fold higher inhibiting effect than Qu. The mixture of flavonoid aglycone and glyceride Qu+Ru showed a lower oxidation stability of lipid

substrate than of Qu alone, however, almost 3-fold higher effect than Ru. Evidently, the addition of Ru to Qu not only didn't improve the antioxidant efficiency of Qu but even led to decrease of the antioxidant potential of the aglycon. Consequently, these both mixtures of Qu+Lu and Qu+Ru show no synergism; furthermore they showed an antagonism between flavonoids. These results manifested that these flavonoid aglycons (Qu and Lu) and glycoside (Ru) must be used as individual antioxidants, not in mixtures (5).

Figure 2. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in presence of 0.1mM Luteolin (Lu), Rutin (Ru), Quercetin (Qu) and of their equimolar (1: 1) binary mixtures (Lu+Qu) and (Qu+Ru)

As we can see from the **Figure 3 and Table 1** the equimolar binary mixtures of flavonoids and TOH demonstrated a synergism between the components: 47% for Qu+TOH, 19% for Lu+TOH and 87% for Ru+TOH.

It is found that the lipid oxidation stability (presented as protection factor, PF in presence of

flavonoids studied) decreases in the order: PF: 22.8 (Qu+TOH) > 19.2 (Ru+TOH) > 15.1 (Lu+TOH) > 8.1 (TOH) > 7.6 (Qu) > 2.1 (Ru) > 1.7 (Lu). New mechanism of action of flavonoids and tocopherol in binary mixtures is presented.

Table 1. Inhibiting efficiency of various binary mixtures of two (1 and 2) antioxidants in equimolar concentrations (0.1mM) and 1:1 ratio, 80°C, during TGSO autoxidation ($IP_c=1.7\pm0.2$ h, $R_c=1.2 \cdot 10^{-6}$ M/s)

Binary mixtures	IP_{1+2} h	IP_1 h	IP_2 h	Synergism/Additi- vism/Antagonism	%Syn %Ant
Qu (1) + Lu (2) (Qu+Lu)	7.5±0.8	9.9±0.9	2.2±0.2	Ant $IP_{1+2} < (IP_1 + IP_2)$	61%
Qu (1) + Ru (2) (Qu+Ru)	8.3±0.8	9.9±0.9	2.7±0.2	Ant $IP_{1+2} < (IP_1 + IP_2)$	52%
Qu (1) + TOH (2) (Qu+TOH)	29.7±1.5	9.9±0.9	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	46%
Lu (1) + TOH (2) (Lu+TOH)	15.1±0.9	2.4±0.2	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	19%
Ru (1) + TOH (2) (Ru+TOH)	24.9±1.5	2.7±0.2	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	87%
CA (1) + TOH (2) (CA+TOH)	20.4±1.5	9.8±0.9	10.5±0.9	Add $IP_{1+2} = (IP_1 + IP_2)$	-
SA (1) + TOH (2) (SA+TOH)	16.1±0.9	5.3±0.5	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	2%
BHT (1) + TOH (2) (BHT + TOH)	21.5±1.5	7.5±0.5	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	19%
TBHQ (1)+ TOH (2) (TBHQ + TOH)	26.1±1.5	7.9±0.5	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	44%
Cum ₂ (1) +TOH (2) (Cum ₂ +TOH)	12.7±0.9	7.1±0.5	10.5±0.9	Ant $IP_{1+2} < (IP_1 + IP_2)$	39%
Cum ₁ (1) +TOH (2) (Cum ₁ +TOH)	14.2±0.9	2.0±0.2	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	14%
Cum ₃ (1) +TOH (2) (Cum ₃ +TOH)	11.8±0.9	1.5±0.2	10.5±0.9	Add $IP_{1+2} = (IP_1 + IP_2)$	-
SA (1) +TOH (2) (SA+TOH)*	45.0±1.0	8.5±0.5	21.0±1.5	Sin $IP_{1+2} > (IP_1 + IP_2)$	52%
M ₁ OH (1) + TOH (2) (M ₁ OH+TOH)	14.8±0.8	1.3±0.2	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	25%
D ₁ (OH) ₂ (1) + TOH (2) (D ₁ (OH) ₂ +TOH)	13.2±0.8	3.2±0.2	10.5±0.9	Add $IP_{1+2} = (IP_1 + IP_2)$	-
M ₂ OH (1) + TOH (2) (M ₂ OH+TOH)	10.3±0.8	1.3±0.2	10.5±0.9	Ant $IP_{1+2} < (IP_1 + IP_2)$	15%
D ₂ (OH) ₂ (1) + TOH (2) (D ₁ (OH) ₂ +TOH)	10.9±0.8	2.0±0.2	10.5±0.9	Ant $IP_{1+2} < (IP_1 + IP_2)$	15%
FA (1) + TOH (2) (FA+TOH)	18.5±0.8	1.9±0.3	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	49%
DFA (1) + TOH (2) (DFA+TOH)	21.5±0.8	2.0±0.3	10.5±0.9	Sin $IP_{1+2} > (IP_1 + IP_2)$	71%

Figure 3. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in presence of 0.1mM Luteolin (Lu), Rutin (Ru), Quercetin (Qu), α -Tocopherol (TOH) and of their equimolar (1:1) binary mixtures (Lu+TOH), (Qu+TOH) and (Ru+TOH)

For that reason the following reactions of binary mixtures of flavonoids (for example quercetin) $\text{Qu}(\text{OH})_2$, α -tocopherol, TOH) and of their radicals - tocopheryl radical ($\text{TO}^\bullet$) and semiquinone radicals $\text{Qu}(\text{OH})\text{O}^\bullet$ with lipid

peroxide radical ($\text{LO}_2^\bullet$) and reactions of homo- and cross-recombination and disproportionation reactions, responsible to different effects observed are presented:

- Reactions of Quercetin ($\text{Qu}(\text{OH})_2$) and its radicals

- Reactions of α -Tocopherol (TOH) and its phenoxyl radical

- Reactions between $\text{Qu}(\text{OH})_2$ and TOH and between their radicals

Scheme 1. The reaction mechanism of action of binary mixtures of quercetin and α -tocopherol

H-atom transfer and cross-disproportionation reactions are responsible for the synergism obtained; however, reaction of cross-recombination is responsible to the antagonism observed. **Table 2** presents results about differences in O-H bond dissociation enthalpies (BDE) of Qu, Lu and Ru and TOH, which are the suitable theoretical descriptor for explanation (and probably for prediction) of the effects of binary mixtures. It is seen that in case when $\Delta\text{BDE} > 0$ it is observed a synergism, and in case of $\Delta\text{BDE} < 0$ an antagonism, respectively.

Figure 4. Structures of cinnamic acids, studied: caffeic acid (CA), ferulic acid (FA) and synaptic acid (SA); and of standard antioxidants studied: butylated hydroxytoluene (BHT) and tret-butylhydroquinone (TBHQ)

Figure 5 presents the kinetics of lipid autooxidation in presence of studied cinnamic

acids and standard antioxidants as individual compounds and in equimolar binary mixtures with TOH.

Figure 5. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in presence of 0.1mM sinapic acid (SA), butylated hydroxytoluene (BHT), tretbutylated hydroquinone (TBHQ), caffeic acid (CA), α -tocopherol (TOH) and of their equimolar (1:1) binary mixtures (SA+TOH), (BHT+TOH), (TBHQ+TOH), and (CA+TOH)

The following order of lipid oxidation stability was found:

(TBHQ+TOH) > (BHT+TOH) > (CA+TOH) > (SA+TOH) > TOH > CA > TBHQ $\geq$ BHT > SA

Tables 1 and 3 present the result of effects observed in these binary mixtures and the differences in BDE_{O-H} with TOH. It is seen that all compounds demonstrated synergism with TOH, only CA manifested additivism with TOH. The strongest inhibiting effects and synergism obtained of the binary mixtures (TBHQ+TOH) and (BHT+TOH) can be explained by the following mechanisms (Schemes 2&3). It is seen that both components of the mixture – BHT (or TBHQ) and TOH can be regenerated during the homo-disproportionation reactions of phenoxyl radicals from BHT (or TBHQ) and TOH formed, as well as during the reactions of cross-disproportionation between their radicals: **Schemes 2, 3.**

Protective effects of binary mixtures of hydroxy-coumarins

Coumarins are important class of oxygen heterocycles, widespread in plant kingdom (11). They have attracted intense interest recently due to their presence in natural sources, and to their possession of diverse pharmacological properties. 4-Methylcoumarins have been found to possess choleric, analgesic, anti-spermatogenic, anti-tubercular and diuretic

properties (6). Polyphenolic coumarins are known to act as antioxidants in biological systems, but it is difficult to distinguish their antioxidant activity from the many other effects they produce in cells. In order to study the possible synergism between two phenolic antioxidants, the antioxidant efficiency and reactivity of tree binary mixtures of coumarins and TOH (Cum_1 +TOH, Cum_2 +TOH and Cum_3 +TOH) were tested and compared. **Figure 6 and Table 1** present results obtained. It is found the higher oxidation stability of the lipid substrate in presence of all binary mixtures in comparison with individual compounds.

The synergism was observed only for the binary mixture of 7,8-dihydroxy-4-methyl coumarin (Cum_1) with α -tocopherol (TOH) Cum_1 +TOH: $IP_{1+2}(14.2) > IP_{Cum_1}(2.0) + IP_{TOH}(10.5)$ and 14 % synergism.

In case of equimolar binary mixture of 6,7-dihydroxy-4-methyl-coumarin (Cum_2) with α -tocopherol (TOH), i.e. Cum_2 +TOH it was found an antagonism between two antioxidants:

$$IP_{1+2}(12.7) < IP_{Cum_2}(7.1) + IP_{TOH}(10.5).$$

In case of 7-hydroxy-4-methyl-coumarin (Cum_3) with α -tocopherol (TOH) Cum_3 +TOH an additivism was observed: $IP_{1+2}(11.8) = IP_{Cum_3}(1.7) + IP_{TOH}(10.5)$.

Regeneration of BHT during homo-dissproponation reaction

Regeneration of TOH during homo-dissproponation reaction

Regeneration of TOH and BHT by H atom transfer reaction -this reaction is reversible

Regeneration of the stronger antioxidant TOH by reaction of cross-dissproportionation

Scheme 2. Mechanism of BHT and TOH in binary mixture with possible regeneration of BHT and TOH

Regeneration of TBHQ during homo-dissproponation reaction

Regeneration of TOH during homo-dissproponation reaction

Regeneration of TOH and TBHQ by H atom transfer reaction -this reaction is reversible

Regeneration of TOH and TBHQ by reaction of cross-dissproportionation

Scheme 3. Mechanism of TBHQ and TOH in binary mixture with possible regeneration of TBHQ and TOH

Tables 2 and 3 present the differences in BDE_{O-H} with TOH.

Table 2. Binary mixtures of bi-phenolic and polyphenolic antioxidants with TOH

$Q(OH)_2$	$Q(OH)_2+TOH$	BDE_{QH_2} ΔBDE_{O-H}	QH_2 regeneration	TOH regeneration	Side reactions
CA	Additivism	$=BDE_{TOH}$ $\Delta BDE = 0$	yes	H atom transfer Cross-disproport	No
7,8-di-OHCum Cum ₁ (OH) ₂	Synergism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	yes	H atom transfer Cross-disproport	No
6,7-di-OHCum Cum ₂ (OH) ₂	Antagonism	$\downarrow BDE_{TOH}$ $\Delta BDE < 0$	yes	H atom transfer	Cross-recombination
FLAVONOIDS					
Qu + Lu	Antagonism	$BDE < 0$	yes	-	Cross-recombination
Qu + Ru	Antagonism	$BDE < 0$	yes	-	Cross-recombination
Qu + TOH	Synergism 46%	$\uparrow BDE_{TOH}$ $BDE > 0$	yes	H atom transfer Cross-disproport	No
Lu + TOH	Synergism 19%	$\uparrow BDE_{TOH}$ $BDE > 0$	yes	H atom transfer Cross-disproport	No
Ru + TOH	Synergism 89%	$\uparrow BDE_{TOH}$ $BDE > 0$	yes	H atom transfer Cross-disproport	No

Table 3. Binary mixtures of monophenolic antioxidants AH with TOH

AH	AH+TOH	BDE_{A-H}	AH regener.	TOH regeneration	Side reactions
BHT	Synergism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	yes	H atom transfer, cross-disproport.	No t-But
SA	Synergism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	no	H atom transfer	Yes +
FA	Synergism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	no	H atom transfer	Yes +
M ₁ OH	Synergism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	no	H atom transfer	Yes +
M ₂ OH	Antagonism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	no	H atom transfer	Yes +++
7-OH-Cum Cum ₃ OH	Additivism	$\uparrow BDE_{TOH}$ $\Delta BDE > 0$	no	H atom transfer	Yes +++

Figure 6. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in presence of 0.1mM 7,8-dihydroxy-4-methyl-coumarin (Cum₁), 6,7-dihydroxy-4-methyl-coumarin (Cum₂), α -tocopherol (TOH) and of their equimolar (1:1) binary mixtures (Cum₁+TOH) and (Cum₂+TOH)

The highest oxidation stability of TGSO in presence of all binary mixtures with α -tocopherol (TOH) may be explained taking into account that the both antioxidants may be regenerated during the oxidation process. It is known that the catecholic moiety of the coumarine molecules allows formation of semiquinone radicals. These semiquinone radicals may regenerate the initial antioxidant molecule during reaction of bimolecular recombination with homo- and cross-disproportionation of semiquinone radicals:

Scheme 4.

These reactions demonstrate that during lipid oxidation process initial molecules of 7,8-dihydroxy-4-methyl coumarin, Cum₁(OH)₂ and TOH are regenerated by different mechanisms and that makes this binary mixture the most powerful antioxidant compositions. It could be seen that the positions of both phenolic groups in coumarin molecule plays an important role in their mechanism of action. In case of 7-hydrpxy-4-methyl- coumarin, Cum₃OH, the observed additivity of the binary mixture (Cum₃+TOH) may be explained with the possible reactions of homo-disproportionation of TO• and cross-disproportionation of phenoxyl radical (Cum₃O•) from monohydroxy coumarin,

and tocopheryl radical (TO•) with regeneration of TOH.

Considering that 4-methylcoumarins, in contrast to many other coumarins, are not metabolized to toxic epoxide intermediates, these results indicate the possible application of these compounds as individual antioxidants and/or in complex binary mixtures.

Protective effects of monomers and dimers

Dehydrozingerone (M₁OH) is a half molecule of curcumin – one of the most powerful antioxidant with a wide spectrum of its biological activity (12). Ginger and curcumin are the well-known spices. Recently synthesized natural-like C2-symmetry hydroxylated biphenyls (dimers) of their corresponding monomers (dehydrozingerone, M₁OH, zingerone, M₂OH and ferulic acid, FA) were selected for this study, because of their interesting biological activity against to neuro-degenerative diseases and melanoma cancer (7).

It is seen (**Figure 7, Table 1**) that dimer of dehydrozingerone, D₁(OH)₂ is able to ensure the higher oxidation stability of lipid substrate, i.e. it has the better protective effect in comparison with corresponding monomer (M₁OH). TOH manifested the best protective effect on lipid autoxidation in comparison with other individual compounds. However, it is evident that AsCPH didn't show any antioxidant activity (potential).

Regeneration of dihydroxy-coumarin $\text{Cum}_1(\text{OH})_2$ by cross-disproportionation reaction Regeneration of $\text{Cum}_1(\text{OH})_2$ by H atom transfer and cross-disproportionation reactions

Regeneration of TOH by cross-disproportionation reactions and H atom transfer

Scheme 4. Mechanism of synergistic effect of binary mixture of 7,8-dihydroxy-4-methyl-coumarin, $\text{Cum}_1(\text{OH})_2$ with α -tocopherol (TOH)

Figure 7. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in absence (control, C) and in presence of 0.1mM monomer M_1OH (dehydrozingerone), of dimer of dehydrozingerone [$D_1(OH)_2$], ascorbyl palmitate (AscPH), α -tocopherol (TOH) and their equimolar binary mixtures (M_1OH +TOH), [$D_1(OH)_2$ +TOH]] and (TOH+AscPH).

The kinetic curve in presence of AscPH is the same as for the control sample. New orders of antioxidant efficiency (as protection factor, PF) and antioxidant reactivity (as inhibition degree, ID) were found for the individual compounds:

PF: TOH (21.2) > $D_1(OH)_2$ (13.5) > M_1OH (3.5) > AscPH (1.0)

ID: TOH (29.3) = $D_1(OH)_2$ (29.3) >> M_1OH (6.3) > AscPH (1.0)

The observed lower antioxidant activity of M_1OH and $D_1(OH)_2$ respect to TOH is expected as a result of the higher bond dissociation enthalpy (BDE) of OH groups of M_1OH and $D_1(OH)_2$ in comparison with those of TOH ($\Delta BDE_{M_1OH}=9.6$ and $\Delta BDE_{D_1(OH)_2}=9.0$ kcal/mol, respectively) (7). Another reason for the lower antioxidant activity of M_1OH and $D_1(OH)_2$ with respect to TOH can be explain with no

possibility of their phenoxyl radicals to be regenerated like TOH during homo-disproportionation reaction.

Data obtained (**Figure 7 and Table 1**) showed that both binary mixtures (M_1OH +TOH and $D_1(OH)_2$ +TOH) lead to the maximal oxidation stability of lipid substrate in comparison with the corresponding individual components. The latest is of importance for the practice, because these binary mixtures are able to ensure the best protective effect on the lipid substrate being oxidized.

PF: $D_1(OH)_2$ +TOH(35.4) > M_1OH +TOH(32.7) > TOH(21.2) > $D_1(OH)_2$ (13.5) >> M_1OH (3.5)

ID: $D_1(OH)_2$ +TOH (29.3) = TOH (29.3) = $D_1(OH)_2$ (29.3) > M_1OH +TOH (25.9) >> M_1OH (6.3)

Figure 8. Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in absence (control, C) and in presence of 0.1mM ascorbyl palmitate (AscPH), of equimolar (1:1) binary mixtures with AscPH of monomer M_1OH +AscPH and of dimer $D_1(OH)_2$ +AscPH, and of their triple (1:1:1) equimolar mixtures with TOH and AscPH of monomer M_1OH +TOH+AscPH) and of dimer $D_1(OH)_2$ +TOH+AscPH).

Effect of equimolar (1:1) binary mixtures of studied compounds with ascorbyl palmitate (AscPH)

Figure 8 and Table 1 showed the data observed for the equimolar binary mixtures of M_1OH , $D_1(OH)_2$ and TOH with AscPH. New orders of antioxidant properties were obtained:

PF: $TOH+AscPH$ (31.5) $\gg$ $D_1(OH)_2+AscPH$ (10.4) $>$ $M_1OH + AscPH$ (6.2) $>$ AscPH (1.0)

ID: $TOH+AscPH$ (29.3) $\gg$ $D_1(OH)_2+AscPH$ (8.8) $>$ $M_1OH+AscPH$ (6.8) $>$ AscPH (1.0)

The highest antioxidant efficiency (PF) was found for binary mixture of TOH + AscPH, which is 3-fold higher than that of $D_1(OH)_2+AscPH$ and 5-fold higher than that of $M_1OH+AscPH$. $D_1(OH)_2$ in binary mixture with AscPH leads to the higher oxidation stability of lipid substrate than in case of $M_1OH+AscPH$.

The binary mixtures of studied compounds ($M_1OH+AscPH$ and $D_1(OH)_2+AscPH$) showed higher protective effect than of AscPH. The latest due to the possible regeneration of M_1OH and $D_1(OH)_2$ from AscPH during the oxidation process:

$M_1O\cdot + AscPH \rightarrow M_1OH + AscP\cdot$ reaction of H atom transfer with monomer regeneration

$M_1O\cdot + AscP\cdot \rightarrow M_1OH + DHAP$ (DHAP, dehydroascorbyl palmitate) cross

disproportionation reaction with monomer regeneration.

Both reactions with monomer regeneration are of importance for the synergism of $M_1OH+AscPH$ mixture obtained, because M_1OH is the stronger antioxidant in comparison with AscPH. Our results confirm again AscPH as synergist, but not as antioxidant during bulk lipid autoxidation. Antagonism was detected only for $D_1(OH)_2+AscPH$ binary mixture that evidenced the lower level of dimer regeneration from AscPH. This result can be explained with the reverse character of H atom transfer reactions:

Probably it is a result of the possible AscPH regeneration in reaction between $AscP\cdot$ and $D_1(OH)_2$ and thus reduces its activity.

Another reason for antagonism obtained is the possible cross recombination reaction between $AscP\cdot$ and $D_1(OH)O\cdot$ to inactive product. The latest reduced the concentration of dimer phenoxyl radical which could be regenerated by H atom transfer from AscPH.

We expected to increase significantly the efficiency of antioxidant compositions, due to the regeneration by different reactions of TOH and M_1OH /or $D_1(OH)_2$ during the oxidative process by preparing ternary mixtures

$M_1OH+TOH+AscPH$ and $D_1(OH)_2+TOH+AscPH$. The protective action of triple mixtures of M_1OH and $D_1(OH)_2$ with TOH and $AscPH$ is presented at **Figure 8 and Table 4**.

PF: $D_1(OH)_2 + TOH + AscPH (35.4) > M_1OH + TOH + AscPH (33.1)$

ID: $D_1(OH)_2 + TOH + AscPH (35.2) > M_1OH + TOH + AscPH (29.3)$

Triple mixture of $D_1(OH)_2$ with TOH and $AscPH$ manifested also a higher antioxidant efficiency and reactivity than the corresponding triple mixture of M_1OH . However, triple mixture of $M_1OH + TOH + AscPH$ showed similar PF as for double mixture of $M_1OH + AscPH$.

PF: $M_1OH+TOH+AscPH (33.1) > M_1OH+TOH (32.7) > TOH(21.2)>> M_1OH+AscPH(6.2) > M_1OH (3.5)$

PF: $D_1(OH)_2+TOH+AscPH(35.4)=D_1(OH)_2+TOH(35.4)>>D_1(OH)_2(13.5)>D_1(OH)_2+AscPH (10.4)$

We proved by HPLC monitoring of the TOH content during the process of lipid autoxidation (9) that the mechanism of synergistic action of binary and triple mixtures of M_1OH and $D_1(OH)_2$ with TOH and $AscPH$ is due to the regeneration of TOH (**Table 4**).

Table 4. Lipid hydroperoxides concentration [$LOOH$] and α -tocopherol (TOH) content determined during lipid autoxidation of triglycerides of sunflower oil (TGSO) at $80^\circ C$.

Abbrev. of mixtures	Time, h	[$LOOH$] average, mM	TOH content, mg/g
$(TOH)_5$	5	4.9±1.1	0.458±0.018
$(TOH)_{10}$	10	11.8±1.2	0.452±0.018
$(TOH)_{15}$	15	18.0±2.0	0.451±0.018
$(TOH)_{20}$	20	25.2±4.3	0.380±0.015
$(TOH)_{25}$	25	33.5±5.5	0.258±0.010
$(TOH)_{30}$	30	143.0±5.0	0.016±0.001
Equimolar binary mixtures with AscPH			
$(TOH+AscPH)_5$	5	2.7±2.5	0.466±0.019
$(TOH+AscPH)_{10}$	10	5.4±4.6	0.454±0.018
$(TOH+AscPH)_{15}$	15	10.2±6.4	0.445±0.018
$(TOH+AscPH)_{20}$	20	14.7±8.8	0.442±0.018
$(TOH+AscPH)_{30}$	30	28.0±12.0	0.440±0.018
$(TOH+AscPH)_{45}$	45	152.0±3.0	0.059±0.002
Equimolar binary mixtures with TOH			
$(M_1OH+TOH)_5$	5	6.3±1.5	0.466±0.019
$(M_1OH +TOH)_{10}$	10	11.0±2.0	0.449±0.018
$(M_1OH +TOH)_{15}$	15	16.2±1.8	0.442±0.018
$(M_1OH +TOH)_{20}$	20	21.6±2.3	0.366±0.015
$(M_1OH +TOH)_{30}$	30	39.2±1.2	0.288±0.012
$(M_1OH +TOH)_{45}$	45	86.0±20.0	0.047±0.002
$(D_1(OH)_2+TOH)_5$	5	5.7±0.9	0.462±0.018
$(D_1(OH)_2+TOH)_{10}$	10	11.3±1.3	0.459±0.018
$(D_1(OH)_2+TOH)_{15}$	15	15.7±2.6	0.452±0.018
$(D_1(OH)_2+TOH)_{20}$	20	23.0±1.0	0.396±0.016
$(D_1(OH)_2+TOH)_{30}$	30	39.4±4.5	0.296±0.012
$(D_1(OH)_2+TOH)_{45}$	45	64.7±2.3	0.049±0.002
Equimolar triple mixtures with TOH and AscPH			
$(M_1OH+TOH+AscPH)_5$	5	2.0±2.0	0.468±0.019
$(M_1OH+TOH+AscPH)_{10}$	10	4.8±4.8	0.467±0.019
$(M_1OH+TOH+AscPH)_{15}$	15	9.5±9.5	0.458±0.018
$(M_1OH+TOH+AscPH)_{20}$	20	13.0±7.0	0.449±0.018
$(M_1OH+TOH+AscPH)_{30}$	30	28.0±7.0	0.329±0.013
$(M_1OH+TOH+AscPH)_{45}$	45	91.0±33.0	0.114±0.005
$(D_1(OH)_2+TOH+AscPH)_5$	5	2.0±2.0	0.468±0.019
$(D_1(OH)_2+TOH+AscPH)_{10}$	10	4.8±4.8	0.466±0.019
$(D_1(OH)_2+TOH+AscPH)_{15}$	15	8.5±4.5	0.462±0.018
$(D_1(OH)_2+TOH+AscPH)_{20}$	20	12.3±6.8	0.459±0.018
$(D_1(OH)_2+TOH+AscPH)_{30}$	30	24.0±10.0	0.426±0.017
$(D_1(OH)_2+TOH+AscPH)_{45}$	45	54.0±17.0	0.379±0.015

Results, obtained about TOH content vs reaction oxidation time were separated in to the following groups:

- ✓ 0.420-0.470 mg/g TOH content; in this group are samples, in which the TOH content is equal to the initial TOH content. Here are TOH_{15} ; $[\text{D}_1(\text{OH})_2 + \text{TOH}]_{15}$; $(\text{M}_1\text{OH} + \text{TOH})_{15}$; $(\text{TOH} + \text{AscPH})_{30}$; $[\text{D}_1(\text{OH})_2 + \text{TOH} + \text{AscPH}]_{30}$ and $(\text{M}_1\text{OH} + \text{TOH} + \text{AscPH})_{20}$;

It was observed that AscPH exhibited a strong effect as synergist and led to 2-fold longer period (30h), in which the initial TOH content was saved, in comparison with the individual TOH (15h). Dimer $\text{D}_1(\text{OH})_2$ and monomer M_1OH in binary mixtures with TOH demonstrated the same initial high TOH content as observed when TOH was used as an individual compound. Monomer M_1OH in ternary mixtures with TOH and AscPH showed the high initial TOH content for a longer period (20h).

- ✓ 0.340-0.420mg/g TOH content; in this group are samples, in which the TOH content is high, but lower than the initial TOH content. Here are TOH_{20} ; $(\text{TOH} + \text{M}_1\text{OH})_{20}$; $[\text{TOH} + \text{D}_1(\text{OH})_2]_{20}$; $[\text{D}_1(\text{OH})_2 + \text{TOH} + \text{AscPH}]_{45}$.

The results obtained after 20h oxidation showed that TOH content is the same for both TOH used alone and for TOH in binary mixtures of monomer M_1OH and dimer $\text{D}_1(\text{OH})_2$. The same TOH content was observed in the ternary mixture of $\text{D}_1(\text{OH})_2 + \text{TOH} + \text{AscPH}$, but after 45h

of lipid autoxidation. The latest result is of great significance in the search for optimal stabilization of lipid substrate.

- ✓ 0.250-0.340 mg/g TOH content; in this group are samples, with moderate TOH content. Here are: $[\text{D}_1(\text{OH})_2 + \text{TOH}]_{30}$; $(\text{M}_1\text{OH} + \text{TOH})_{30}$; $(\text{M}_1\text{OH} + \text{TOH} + \text{AscPH})_{30}$.

It was observed that, after 30h oxidation of lipid substrate, the same TOH content for binary mixtures of M_1OH and $\text{D}_1(\text{OH})_2$ with TOH were obtained, which is equal to those of the mixture of $\text{M}_1\text{OH} + \text{TOH} + \text{AscPH}$.

- ✓ 0.040-0.120 mg/g TOH content; in this group samples, with the lowest TOH content.

Only ternary mixture of $(\text{M}_1\text{OH} + \text{TOH} + \text{AscPH})_{45}$ to this group with low content of TOH. For comparison, the ternary mixture of $\text{D}_1(\text{OH})_2 + \text{TOH} + \text{AscPH}$, after 45h oxidation reaction time, manifested much higher TOH content, equal to the initial TOH content. It must be noted that the binary mixtures of $(\text{TOH} + \text{AscPH})_{45}$, $[\text{D}_1(\text{OH})_2 + \text{TOH}]_{45}$; and $(\text{M}_1\text{OH} + \text{TOH})_{45}$ showed the same TOH content after 45h oxidation time of lipid substrate, but much lower in comparison with the ternary mixture of M_1OH .

Complex mechanisms of TOH regeneration in binary and triple mixtures are presented at **Figure 9 (a-d)**.

Figure 9a. Complex mechanism of TOH regeneration during lipid autoxidation added as individual antioxidant (Path a) and in double mixtures with $\text{D}_1(\text{OH})_2$ (Path b), M_1OH (Path c), and AscPH (Path d)

- Path a): a1)** H-atom transfer from TOH to $\text{LO}_2^\bullet$ (the key reaction of inhibited oxidation);
a2) Homo-disproportionation of tocopheryl radicals ($\text{TO}^\bullet$) with TOH regeneration;
Path b): b1) H-atom transfer from TOH to $\text{D}_1(\text{OH})\text{O}^\bullet$ with regeneration of $\text{D}_1(\text{OH})_2$;
b2) H-atom transfer from $\text{D}_1(\text{OH})_2$ to $\text{TO}^\bullet$ with regeneration of TOH;
Path c): c1) H-atom transfer from M_1OH to $\text{TO}^\bullet$ with regeneration of TOH;
c2) H-atom transfer from TOH to $\text{M}_1\text{O}^\bullet$ with regeneration of M_1OH ;
Path d): d1) Cross-disproportionation between $\text{TO}^\bullet$ and $\text{AscP}^\bullet$ with regeneration of TOH
 And DHAP – dehydroascorbylpalmitate formation;
d2) H-atom transfer from AscPH to $\text{TO}^\bullet$ with regeneration of TOH.

Observed synergism of $\text{M}_1\text{OH}+\text{TOH}$ (32.3 %) is due to the possible regeneration of TOH, as the strongest antioxidant, by reaction of H atom

transfer from M_1OH to the tocopheryl radical, $\text{TO}^\bullet$.

Figure 9b. Complex mechanism of possible side reactions tocopherol TOH and its tocopheryl radical $\text{TO}^\bullet$ resulting to decrease of TOH activity

- Path a): a1)** Additional generation of free radicals ($\text{LO}_2^\bullet$) from TOH in reaction with lipid substrate (LH); (at high TOH concentration - activity of TOH decreases);
a2) TOH increases decomposition of LOOH into free radicals (side reaction, reducing activity of TOH);
Path b): b1) Cross-recombination of tocopheryl radical ($\text{TO}^\bullet$) and $\text{M}_1\text{O}^\bullet$ (formation of inactive products);
b2) Homo-recombination of two tocopheryl radicals ($\text{TO}^\bullet$) to inactive products;
Path c): c1) H-atom transfer from TOH to $\text{D}_1(\text{OH})_2^\bullet$ with regeneration of $\text{D}_1(\text{OH})_2$;
c2) Cross-disproportionation reaction between $\text{TO}^\bullet$ and $\text{D}_1(\text{OH})_2^\bullet$ with regeneration of $\text{D}_1(\text{OH})_2$;
Path d): d1) Cross-disproportionation reaction between $\text{TO}^\bullet$ and $\text{M}_1\text{O}^\bullet$ with regeneration of M_1OH ;

Figure 9c. Complex mechanism of synergism obtained between components in triple mixture $\text{M}_1\text{OH} + \text{TOH} + \text{AscPH}$

Main reactions, responsible to the synergism obtained:

- a1) H-atom transfer from TOH to $\text{LO}_2^\bullet$ (the key reaction of inhibited oxidation)
- b1) H-atom transfer from M_1OH to $\text{TO}^\bullet$ with regeneration of TOH;
- c1) H-atom transfer from AscPH to $\text{M}_1\text{O}^\bullet$ with regeneration of M_1OH ;
- d1) H-atom transfer from AscPH to $\text{TO}^\bullet$ with regeneration of TOH;

Additional reactions:

- a2) Decomposition of LOOH by tocopheryl radical ($\text{TO}^\bullet$) to $\text{LO}_2^\bullet$ (decreasing TOH activity)
- b2) H-atom transfer from TOH to $\text{M}_1\text{O}^\bullet$ with regeneration of M_1OH ;
- c2) H-atom transfer from AscPH to $\text{M}_1\text{O}^\bullet$ with regeneration of M_1OH ;
- d2) H-atom transfer from TOH to $\text{AscP}^\bullet$ with regeneration of AscPH.

Figure 9d .Complex mechanism of additivity obtained between components in triple mixture $\text{D}_1(\text{OH})_2 + \text{TOH} + \text{AscPH}$

Main reactions, responsible to the synergism obtained:

- a1) H-atom transfer from TOH to $\text{LO}_2^\bullet$ (the key reaction of inhibited oxidation)
- b1) H-atom transfer from AscPH to $\text{TO}^\bullet$ with regeneration of TOH;
- c1) H-atom transfer from $\text{D}_1(\text{OH})_2$ to AscPH with regeneration of AscPH;
- d1) H-atom transfer from TOH to $\text{D}_1(\text{OH})\text{O}^\bullet$ with regeneration of $\text{D}_1(\text{OH})_2$;

Additional reactions:

- a2) Decomposition of LOOH by tocopheryl radical ($\text{TO}^\bullet$) to $\text{LO}_2^\bullet$ (decreasing TOH activity)
- b2) H-atom transfer from TOH to $\text{AscP}^\bullet$ with regeneration of AscPH;
- c2) H-atom transfer from AscPH to $\text{D}_1(\text{OH})\text{O}^\bullet$ with regeneration of $\text{D}_1(\text{OH})_2$;
- d2) H-atom transfer from TOH to $\text{D}_1(\text{OH})\text{O}^\bullet$ with regeneration of $\text{D}_1(\text{OH})_2$.

CONCLUDING REMARKS

Antioxidant activity is capacity of the compound to shorten the oxidation chain length as a result of its reaction with peroxy radicals. For that reason we mean as antioxidant activity the chain-breaking activity of the compounds.

Synergism between individual components in binary mixtures is explained with differences in $\text{BDE}_{\text{O-H}}$ and by H atom abstraction and cross-disproportionation reaction, leading to regeneration of the most powerful antioxidant. Additivity is found for antioxidants with close $\text{BDE}_{\text{O-H}}$ and similar power.

Antagonism is found for antioxidants with lower $\text{BDE}_{\text{O-H}}$ than of TOH and when regeneration of antioxidants is not possible during the reaction of cross-recombination of both phenoxyl radicals.

Structural characteristics of the complex system: oxidizing substrate-antioxidant must be considered. On the basis of this comparative analysis, the most effective individual

antioxidants and binary mixtures were proposed for the highest and optimal lipid oxidation stability. The latest is of importance for the practice.

REFERENCES

1. E.B. Burlakova, Bioantioxidants, *Russ. Chem. J.*, 51, 3-12, 2007.
2. V.D. Kancheva, & O.T. Kasaikina, Bioantioxidants - a chemical base of their antioxidant activity and beneficial effect on human health, *Current Medicinal Chemistry*, 20, 4784-4805, 2013.
3. V.Kancheva, *Oxidative Stress and Lipid Oxidation*, in: "Antioxidants - Prevention and Healthy Aging", Ed. by F.Ribarova, SIMELPRESS Publ., Sofia, Bulgaria, Chapter 3, 233-238, 2010.
4. V.D.Kancheva, Phenolic Antioxidants of Natural Origin – Structure Activity Relationship and their Beneficial Effect on

- Human Health. In: "Phytochemicals and Human Health: Pharmacological and Molecular Aspects", Nova Science Publishers Inc., USA, Ed. A.A.Farooqui, 2011, Chapter I, 1-45
5. V.D.Kancheva, R.Taskova, I.Totseva, N.Handjieva; Antioxidant Activity of Extracts, Fractions and Flavonoid Constituents from *Carthamus lanatus* L., *Riv. Ital. delle Sost. Grasse*, 84, 77-86, 2007.
 6. V.D.Kancheva, L.Saso, P.V.Boranova, M.K.Pandey, Sh.Malhota, J.T.Nechev, A.K.Prasad, M.B.Georgieva, A.L.DePass, V.S.Parmar, Structure-Activity Relationship of Some Dihydroxy Coumarins. Correlation between Experimental and Theoretical Data and Synergistic Effect. *BIOCHIMIE*, 92 (9), 1089-1100, 2010.
 7. V.D.Kancheva, A. Slavova-Kasakova, D.Fabbri, S.Angelova, M.A.Dettori, J.Nechev, G.Delogu, Antiradical and Antioxidant Activities of New Natural-like Biphenyls of Zingerone, Dehydrozingerone and Ferulic Acid, *Comp. Rend. Acad. Bulg. Sci.*, 66 (3), 361-369, 2012.
 8. V.D.Kancheva, A.Slavova-Kazakova, D.Fabbri, M.A. Dettori, G. Delogu, M.Janek, R. Amarowicz, Protective effects of equimolar mixtures of dehydrozingerone and its dimer with α -tocopherol and/or ascorbylpalmitate during lipid autoxidation, *Food Chem*, 157, 263-274, 2014.
 9. V.D.Kancheva, Phenolic Antioxidants – Radical Scavenging and Chain Breaking Activities. Comparable study. *Eur J Lipid Sci Technol.*, 111 (11), 1072-1089, 2009.
 10. R.Torres, F.Faini, B.Modak, F.Urbina, C.Labbe, Y.Guerro, Antioxidant activity of coumarins and flavonoids from the resinous exudates of *Haplopappus multifolines*, *Phytochemistry*, 67, 984-987, 2006.
 11. E.T.Denisov, T.G. Denisova, Handbook of antioxidants, bond dissociation energies, rate constants, activation energies and enthalpies of reactions. New York: CRS Press, 2001.
 12. A.Marchiani, C.Rozzo, A. Fadda, G.Delogu, R. Ruzza, R. Curcumin and curcumin-liked molecules from spice to drugs. *Current Medicinal Chemistry*, 21, 204–222, 2014.