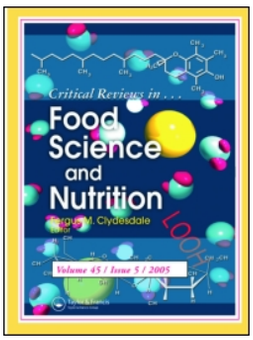

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Dietary antioxidant synergy in chemical and biological systems

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ABSTRACT

Antioxidant (AOX) synergies have been much reported in chemical ("test-tube" based assays focusing on pure chemicals), biological (tissue culture, animal and clinical models), and food systems during the past decade. Tentative synergies differ from each other due to the composition of AOX and the quantification methods. Regeneration mechanism responsible for synergy in chemical systems has been discussed. Solvent effects could contribute to the artifacts of synergy observed in the chemical models. Synergy in chemical models may hardly be relevant to biological systems that have been much less studied. Apparent discrepancies exist in understanding the molecular mechanisms in both chemical and biological systems. This review discusses diverse variables associated with AOX synergy and molecular scenarios for explanation. Future research to better utilize the synergy is suggested.

KEYWORDS

Antioxidant; synergy; chemical system; biological system; food; mode of action

Introduction

In biological systems, dietary antioxidants (AOXs) have a broad spectrum of nutraceutical/pharmacological potential for prevention and/or treatment of various oxidative stress (OS) induced chronic diseases, including certain types of cancers, cardiovascular diseases, diabetes, and Alzheimer's disease (Valko et al., 2007; Sen and Chakraborty, 2011). In food fortification and preservation, dietary AOXs can prevent/retard deterioration of the oxidizable foods (e.g., lipid oxidation and discoloration of fruits and vegetables), extending their shelf life (Osada et al., 2001; Vinson et al., 2002; Guimaraes et al., 2010; Karpińska-Tymoszczyk, 2014; Calligaris et al., 2015).

A stable mixture of two or more AOX individuals (purified compounds and/or crude extracts) creates a "complex network." The cooperative action of AOX individuals displays a greater AOX effect than the sum of that of the individuals acquired independently. This is termed AOX synergy (Uri, 1961; Yeum et al., 2009; Hidalgo et al., 2010). Synergistic effect can be mathematically quantified by the difference between the experimental value and the theoretical value (obtained as the summation of AOX capacities of individual AOXs). In the case of a binary mixture of two substances A and B, difference in AOX activity = AOX capacity value of the mixture − [(AOX capacity value $_A$ × concentration $_A$) + (AOX capacity value $_B$ × concentration $_B$)]. A positive value indicates an AOX synergy. Synergy effect (%) is estimated by the difference divided by theoretical value in this review. AOX synergies are desirable for achieving an expected level of AOX capacity in biological or food systems, while minimizing the usage quantities of individuals.

Over the last decade, numerous AOX synergies have been identified from binary, ternary, quaternary, and quinary mixtures of diverse substances. The individuals included purified components [e.g., α, γ-tocopherols (TOH), ascorbic acid (AA)] (Becker et al., 2007; Yeum et al., 2009; Hazewindus et al., 2012; Thoo et al., 2013; Kamal−Eldin and Budilarto, 2014; Moser et al., 2014), endogenous enzymes [e.g., glutathione (GSH), superoxide dismutase (SOD), catalase (CAT)] (Pereira et al., 2013; Asokkumar et al., 2014), polyphenols [e.g., phenolic acids like gallic acid (GA), caffeic acid (CA); flavonoids (FOHs) like resveratrol (RES), rutin (R), catechin (CATE), epicatechin (EC), and (-)-epigallocatechin gallate (EGCG)] (Cuvelier et al., 2000; Becker et al., 2007; Liang et al., 2009; Cuvelier et al., 2000; Hidalgo et al., 2010; Peinado et al., 2010; González and Nazareno, 2011; Yin et al., 2012; Pereira et al., 2013; Asokkumar et al., 2014; Skroza et al., 2015), synthetic AOXs [e.g., butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)] (Romano et al., 2009; Fernández-Alvarez et al., 2014), and herbal and synthetic AOX drugs (e.g., fenofibrate and famotidine) (Asokkumar et al., 2014). The individuals also included complex mixtures, such as mixtures of diverse gains (wheat, oat, rye, wheat bran, soy, maize) for bread production (Serpen et al., 2012), crude extracts of foodstuff (e.g., herbal tea, fruits, legumes) (Romano et al., 2009; Wang et al., 2011; Yin et al., 2012), and crude extracts of medicinal plants [e.g., *Carica papaya* leaf (CPL), *Azadarichta indica* leaf (AIL)] (Thoo et al., 2010; Guimaraes et al., 2011; Irondi et al., 2012; Pereira et al., 2014).

Synergy formulations showed great variability in structure feature, concentration, and volume ratio of the individual AOX, as well as estimation methods (Zhou et al., 2005; Becker et al., 2007). Most of the AOX synergies have been observed in chemical systems (homogenous chemical solutions). DPPH, ABTS, and FRAP assays are the most used quantification methods in predicting synergy, the accuracy of which may be affected by possible artifacts (e.g., solvent effect-derived artifact). Synergy behaviors in these systems may be hardly relevant to those in biological systems (e.g., cultured cells, animals, and humans), though

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tremendous amounts of work have been done on the former. This requires a better understanding of the mechanisms of AOX synergy for their applications under physiological circumstances or in food matrices. This review summarizes the current understanding of synergy mechanisms in chemical and biological systems, discusses possible factors influencing the synergy estimation, and suggests research to better utilize AOX synergy in diverse systems.

Synergy in chemical systems

Chemical systems for AOX synergy quantification were designed to reflect the actual situation in food and biological (e.g., human) systems, and tend to be quick, handy, and less costly for synergy qualification (Huang et al., 2005; Prior et al., 2005; Schaich, 2006; Nenadids et al., 2007; Frankel and Finley, 2008; Magalhaes et al., 2008; Moon and Shibamoto, 2009; Baldarinath, 2010; Niki, 2010; Takashima et al., 2012; Magalhães et al., 2014). AOX synergies identified in chemical models showed great variability in the analytical methods and experimental conditions (e.g., AOX composition, oxidative stress inducer, test medium, and solubility) (Table 1).

Quantification methods

A wide variety of analytical techniques has been applied to measure the synergy (Table 1). They can be broadly classified into spectrophotometric and nonspectrophotometric assays. Approximately, 85% of assays listed in Table 1 belong to the former assay.

Spectrophotometric assays

Numerous spectrophotometric assays have been applied to synergy quantification based on different principles. The most popular methods are 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS), thiobarbituric acid reactive substances (TBARS), ferric reducing ability of plasma (FRAP), and oxygen radical absorbance capacity (ORAC) assays. Assays based on free radical scavenging activity (FRSA) of the mixtures are typically carried out in homogeneous solutions containing free radicals, such as DPPH·, $ABTS^{\cdot+}$, superoxide radicals ($O^{2-}\cdot$), and tert-butoxyl radicals (*t*-BuOOH·) (Fujisawa et al., 2006; Chen and Blumberg, 2008; Irondi et al., 2012; Nalewajko-Sieliwoniuk et al., 2012; Ajuwon et al., 2013; Pereira et al., 2013; Wang et al., 2013; Bai et al., 2014; Pereira et al., 2014; Vijayalakshmi et al., 2014; Skroza et al., 2015; Wang et al., 2015a).

ABTS and DPPH are favored because of their low cost and user-friendliness. However, Floegel et al. (2011) pointed out DPPH assay may underestimate the AOX capacity of highly pigmented samples compared with ABTS assays. Another advantage of the ABTS assay is its applicability to both hydrophilic and lipophilic AOX systems, while DPPH is applicable to hydrophobic AOX systems (Floegel et al., 2011). Various AOX mixtures under acidic conditions (PH 3.6) can nonspecifically reduce ferric ion complex (Fe^{3+}-TPTZ) to ferrous ion 2,4,6-tris (2-pyridyl)-s-triazine (Fe^{2+}-TPTZ), as determined by the FRAP assay (Chen and Blumberg, 2008; Wang et al., 2011; Irondi et al., 2012; Skroza et al., 2015; Wang et al., 2015a; 2015b). However, FRAP assay is only feasible with hydrophilic AOXs. Although both hydrophilic and lipophilic AOXs fit into ORAC test conditions, the reported ORAC values (in Table 1) reflect only hydrophilic nature of the synergies (Chen and Blumberg, 2008; Wang et al., 2011).

Synergies on inhibiting lipid oxidations have been commonly characterized by UV-vis/fluorescence-based methods. Among them are the methods that capture the lag phase before the onset of oxidation (Celik et al., 2015), conjugated diene formation in liposome or micelle-based systems (Liang et al., 2009; Almeida et al., 2011), and the oxidation kinetics on the lipophilic fluorescent marker BODIPY C11 581/591 (BODIPY11). Oxidation can be initiated by water-soluble initiators, such as 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH), cupric ion (Cu^{2+}), nicotinamideadenine dinucleotide phosphate tetra-sodium salt ($NADP^{+}$), and iron ion (Fe^{2+}), and lipophilic initiators, such as 2,20-azobis (4-methoxy-2, 4-dimethylvaleronitrile) (MeO-AMVN) (Shi et al., 2007; Liang et al., 2009; Yin et al., 2012).

Nonspectrophotometric assays

There are some other less commonly used approaches for synergy estimation. Briggs–Rauscher (BR) reaction was used to identify rosemary extract (RE) synergized with CATE at the ratio of 1:1 (Skroza et al., 2015). pBluescript II SK(-) DNA oxidation assay was employed to identify the fraction of acetone crude extracts of *Potentilla fruticosa* leaves synergized with *Ginkgo biloba* extracts at a ratio of 5:1, using agarose gel electro-phoretic patterns (Peinado et al., 2010). Methyl methacrylate (MMA) polymerization initiated by 2,2′-azobisisobutyronitrile (AIBN) was used to identify that δ-TOH synergized with (-)-epicatechin gallate (EG) or (-)-epigallocatechin gallate (EGCG), as measured by differential scanning calorimetry (DSC) (Fujisawa et al., 2006; Kadoma et al., 2006; Zou and Akoh, 2015). MMA polymerization initiated by thermal decomposition of benzoyl peroxide (an oxygen-centered radical, PhCOO·) was used to determine the synergy of TOH with L-ascorbyl 2,6-dibutyrate (ASDB, an ascorbate derivative) via induction period (Becker et al., 2007). Pulse voltammetry (voltammetric determination) was applied to reveal that AA synergized with water-soluble bioactives (cysteine, acetylcysteine, glutathione, and methionine), which contribute to the AOX protection system of the body. Differential voltammograms of oxygen reductions on copper cathode, and Fe^{2+} reduction on a platinum cathode, in NaCl solution (0.1 M), were employed (Shapoval and Kruglyak, 2011). Electron spin resonance (ESR) spectroscopy was applied to measure the reduction percentage of Fremy's salt radicals, and carbon-centered 1-hydroxyethyl radicals generated in a Fenton-type reaction (Becker et al., 2007; Yin et al., 2012; Celik et al., 2015). The mechanisms of the above-mentioned methods in relation to synergy estimation are still to be better understood.

Factors affecting the synergy effect in chemical models

Methods

Observed synergies in the chemical models are rather method-sensitive. For example, Chen and Blumberg (2008) showed that interactions between almond skin polyphenols (ASP) and AA were synergistic in assays of free radicals (DPPH, hydroxyl,

Table 1. Synergy representatives in chemical systems (2005–2015).

Assayed mixtures [Identified synergy] *References*		Antioxidant capacity assays involved				Mode of action
Binary mixture of RE with pure phenolics (GA, CAT, Q, CA) [CATE + RE, 1:1] *Skroza et al.(2015)*	*Assay*	BR reaction	FRAP	DPPH		TBD
	Inducer	BR oscillating	TPTZ–Fe^{3+}	DPPH·		
	Test Cond.	30°C, 60 min	37°C, pH 3.6, 4, 8, 20 min	RT, dark, 30, 60 min		
	Measurement	Abs: 620 nm	Abs: 592 nm	Abs: 492 nm		
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic		
	Synergy effect	53%	1%	2%		
Binary mixture of ACEPg with EGb, ACEPg fractions (C-3 and D-2), pure phenolics (CATE, Q, FA, EA, CA, R, HYPE, KAEM, ISOR) [CATE + ISOR, 1:1] *Wang et al. (2015a)*	*Assay*	ABTS	FRAP	DPPH		TBD
	Inducer	ABTS·	TPTZ–Fe^{3+}	DPPH·		
	Test Cond.	RT, pH 7.4, 6 min	37°C, pH 3.6, dark, 30 min	RT, dark, 30 min		
	Measurement	Abs: 734 nm	Abs: 593 nm	Abs: 517 nm		
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic		
	Synergy effect	69%	16%	29%		
Binary mixture of RS with SU [RS + SU, 1:1] *Wang et al. (2015a)*	*Assay*	ORAC	FRAP	DPPH		TBD
	Inducer	AAPH	TPTZ–Fe^{3+}	DPPH·		
	Test Cond.	37°C, 60 min	37°C, pH 3.6, 12 min	RT, dark, 30 min		
	Measurement	Fluorescence ex/em: 535/485 nm	Abs: 593 nm	Abs: 517 nm		
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic		
	Synergy effect	13%	33%	5%		
Binary mixtures of insoluble fractions of grains (IW, IR, IO) with beverage (GT, PJ, ESP), pure antioxidants (EGCG, EC) [IR + ESP, 100 μL + 100 μL ID + ESP, 100 μL + 100 μL IW + EGCG, 5 mg/mL + 100 μM IR + EC, 5 mg/mL + 100 μM] *Celik et al. (2015)*	*Assay*	Lipid oxidation inhibition	ESR spectroscopy			TBD
	Inducer	Fe(III)	Fremy's salt radicals			
	Solubility	Hydro/lipophilic	Hydro/lipophilic			
	Synergy effect	580% 221% 116% 172%	73% 119% 291% 124%			
Binary mixture of β-carotene with CGA [β-carotene + CGA] *Vijayalakshmi et al. (2014)*	*Assay*	Oxidation rate				β-carotene regenerated CGA; β-carotene and CGA formed a stable intermolecular complex
	Inducer	t-BuOOH				
	Test Cond.	RT, irritation				
	Measurement	Abs: 328 nm, CGA 491 nm, β-carotene)				
	Solubility	Hydrophilic				
	Synergy effect	27% (oxidation rate) 35% (quantum yield)				
Ternary mixtures of AR with BO and MT [AR + BO + MT, 15% + 15% + 35%] *Pereira et al. (2014)*	*Assay*	TBARS	Reducing power	β-carotene bleaching	DPPH	TBD
	Inducer	Fe^{2+}	Fe^{3+}	β-carotene	DPPH·	
	Test Cond.	37°C, pH 7.4	50°C, pH 6.6	50°C	RT, 30 min	
	Measurement	Abs: 532 nm	Abs: 690 nm	Abs: 470 nm	Abs: 515 nm	
	Solubility	Lipophilic	Lipophilic	Lipophilic	Lipophilic	
	Synergy effect	88%	75%	67%	89%	
Binary mixtures of CAPE with trolox [CAPE + Trolox, 0.5 μM + 0.5 μM] *Bai et al. (2014)*	*Assay*	HO·	O_2^{-}·	DPPH		TBD

(*Continued on next page*)

Table 1. (*Continued*)

Assayed mixtures [Identified synergy] *References*	Antioxidant capacity assays involved				Mode of action
	Inducer	$CoCl_2$-EDTA H_2O_2	$FeSO_4{\cdot}7H_2O$	DPPH·	
	Test Cond.	30 s	15 s	RT, dark, 20 min	
	Measurement	Chemi -luminescence	Chemi-luminescence	Abs: 517 nm	
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic	
	Synergy effect	134%	197%	50%	
Binary mixture of ascorbate with Q and naringin [Q+ascorbate, naringin + ascorbate]	*Assay*	DPPH			TBD
González and Nazareno (2011)					
	Inducer	DPPH·			
	Test Cond.	27°C, 1–180 min			
	Measurement	Abs: 517 nm			
	Solubility	Hydrophilic			
	Synergy	118%			
	effect	133%			
Binary mixtures of extract of 4 fruits with 4 vegetables, 3 leguminous seeds[a][RS + AD, 1:1]	*Assay*	ORAC	DPPH	FRAP	TBD
Wang et al. (2011)					
	Inducer	AAPH	DPPH·	$TPTZ–Fe^{3+}$	
	Test Cond.	37°C, 60 min	RT, dark, 30 min	37°C, pH 3.6, 12 min	
	Measurement	Fluorescence ex/em: 535/485 nm	Abs: 517 nm	Abs: 593 nm	
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic	
	Synergy effect	0.8%	24%	19%	
Binary mixtures of glucose/ fructose mixture with grapes must, Q	*Assay*	pBluescript II SK(−) DNA oxidation	β-carotene bleaching	Deoxy-ribose assay	TBD
	Inducer	$FeSO_4.H_2O_2$	H_2O_2, 50°C	Fe^{3+} -EDTA Fe^{3+}	
	Test Cond.	37°C, pH 7.4	20, 40, 60, 90 min, pH 7.0	37°C, pH 7.4	
	Measurement	Agarose gel electro-phototetic patterns	Abs: 470 nm	Abs: 520 nm	
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic	
[Glucose/fructose mixture + grape must]	*Synergy effect*	23%	48%	50%	
Peinado et al. (2010)					
Binary mixtures of AA with ASP-M and ASP-G [ASP-M + AA]	*Assay*	Radical scavenging (4 radicals)	FRAP	ORAC	TBD
	Inducer	DPPH·, ONOO-, HOCl, O^{2-}.	$TPTZ–Fe^{3+}$	AAPH	
Chen and Blumberg (2008)	*Test Cond.*	RT, dark, 30 min; RT, 5 min; RT, 5 min; RT, 10 min; xanthine/xanthine oxidase system	RT	37°C, 60 min	
	Measurement	Abs: 520 nm Fluorescence: ex/em: 530/485 nm Abs: 420 nm Abs: 560 nm	Abs: 593 nm	Fluorescence ex/em: 520/485 nm	
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic	
	Synergy effect	6% (DPPH·) 84% (ONOO-) 8% (HOCl) 35% (O^{2-}·)	78%	N.D.	
Ternary mixtures of α-TOH with AA, and GOHs (EC, EGC, ECG, EGCG, GA) [GOH + α-TOH + AA]	*Assay*	Lid oxidation			GOH recycled α-TOH; AA recycled GOH
	Inducer	AAPH			
Dai et al. (2008)	*Test Cond.*	37°C, pH 7.4, SDS micelles			
	Measurement	HPLC			
	Solubility	Hydrophilic			
	Synergy effect	28%			
	Assay	MMA polymerization			Flavonoids regenerated α-TOH; In ASDB/

(*Continued on next page*)

Table 1. (*Continued*)

Assayed mixtures [Identified synergy] *References*	Antioxidant capacity assays involved			Mode of action
Binary mixtures of α-TOH or ASDB with EC, EGC, ECG, EGCG, and MG				flavonoid mixture, ASDB partially regenerated flavonoids
[ASDB + MG]	*Inducer*	AIBN		
Fujisawa et al. (2006)	*Test Cond.*	70°C		
	Measurement	DSC		
	Solubility	Hydrophilic		
	Synergy effect	65%		
Binary mixtures of α, β, δ-TOH with ASDB, EC, and EGCG	*Assay*	MMA polymerization		Lower steric effect of δ-TOH (not α-, β-TOH) resulted in partial regeneration between δ-TOH and EC
[δ-TOH + EC]	*Inducer*	Oxygen-centered radical (PhCOO·)		
Kadoma et al. (2006)	*Test Cond.*	70°C, nearly anaerobic conditions		
	Measurement	DSC		
	Solubility	Lipophilic		
	Synergy effect	60%		
Binary mixtures of AA with cysteine, acetylcysteine, glutathione, and methionine	*Assay*	Voltammetry assay		TBD
[AA + acetylcysteine, 1:2, AA + cysteine, 1:2]	*Inducer*	Electro-Fenton		
Shapoval and Kruglyak (2011)	*Test Cond.*	20°C, pH 7.4		
	Measurement	Current–voltage (Fe^{2+} reduction, oxygen reduction)		
	Solubility	Hydrophilic		
	Synergy effect	52% (AR); 20% (AOX) 61% (AR); 18% (AOX)		
Binary or ternary mixtures of α-TOH with β-carotene and AP	*Assay*	Lipid oxidation		AP regenerated α-TOH; α-TOH protected β-carotene oxidation
[α-TOH + β-carotene]	*Inducer*	N/A		
Karabulut (2010)	*Test Cond.*	In butter oil triacylglycerols		
	Measurement	Iodometric		
	Solubility	Lipo/hydrophilic		
	Synergy effect	28%		
Binary mixtures of 11 flavonoids[b]	*Assay*	FRAP	DPPH	TBD
	Inducer	TPTZ–Fe^{3+}	DPPH·	
	Test Cond.	37°C, pH 3.6, 15 min	RT, dark, 60 min,	
	Measurement	Abs: 593 nm	Abs: 517 nm	
	Solubility	Hydrophilic	Hydrophilic	
[ECAT + Q-3-G	*Synergy effect*	7%	N/A	
K + MY		6%	N/A	
CY + MY		7%	0.1%	
K + Q		5%	1%	
K + Q-3-G]		3%	2%	
Hidalgo et al. (2010)				
Binary mixtures of α-TOH with GTE, ECAT, and CAT	*Assay*	Oxygen consumption	Lipid oxidation	ESR spectroscopy	Structure complementary or physical barriers between AOXs and the lipid derived radicals
	Inducer	MMb	AAPH	N/A	
	Test Cond.	25°C, pH = 6.8, in methyl linoleate o/w emulsion	34°C, pH7.4, in soybean PC liposomes	70°C, in bulk sunflower oil	
	Measurement	Electrochemical	Abs: 234 nm	ESR signal	
	Solubility	Lipophilic	Lipophilic	Lipophilic	
[α-TOH + GTE, 2:1]	*Synergy effect*	2500%	33%	55%	
Yin et al. (2012)					
Binary mixtures of Q with α-TOH, R, and astaxanthin	*Assay*	Oxygen consumption	Lipid oxidation	ESR spectroscopy	

(*Continued on next page*)

Table 1. (*Continued*)

Assayed mixtures [Identified synergy] *References*	Antioxidant capacity assays involved			Mode of action
[α-TOH + Q, 1:1] *Becker et al. (2007)*				AOXs differ in solubility and phase distributions near or at the interface
Inducer	MMb	AAPH	Thermal	
Test Cond.	25°C, pH 6.8. in methyl linoleate o/w emulsion	37°C, pH 7.4 in soybean PC liposomes	70°C, in sunflower oil	
Measurement	Electrochemical: oxygen electrode	Abs: 234 nm	PBN-adducts	
Solubility	Hydro/lipophilic	Hydro/lipophilic	Hydro/lipophilic	
Synergy effect	102%	155%	50%	
Binary mixtures of AA or α-TOH with lycopene and β-carotene [α-TOH + lycopene, 1 μM + 12.5 μM] *Shi et al. (2007)*	*Assay*	Lipid oxidation		TBD
Inducer	AMVN			
Test Cond.	20°C, LAME			
Measurement	Abs: 234 nm			
Solubility	Lipophilic			
Synergy effect	30%			
Binary mixtures of GSH with 10 flavonoids [c][L-7-O-G + GSH, 3.125 μM + 25 μM] *Pereira et al. (2013)*	*Assay*	DPPH		A catechol group located in the B ring of flavonols possibly led to synergy except for an –OH co-located at C6
Inducer	DPPH.			
Test Cond.	RT, 30 min			
Measurement	Abs: 515 nm			
Solubility	Hydrophilic			
Synergy effect	105%			
Binary mixtures of BHT with BHA [RE + BHT, 10 μg/mL + 4.5 g/mL] *Romano et al. (2009)*	*Assay*	DPPH		Heterologous activation of an oxydryle group of an AOX molecule by another AOX
Inducer	DPPH·			
Test Cond.	RT, dark			
Measurement	Abs: 492 nm, 515 nm			
Solubility	Hydrophilic			
Synergy effect	115%			
Binary mixtures of baicalin with β-carotene [baicalin + β-carotene, 1:1] *Liang et al. (2009)*	*Assay*	Lipid oxidation	ABTS/TEAC	Baicalin regenerated β-carotene in the methanol/chloroform solvent (1:9, v/v)
Inducer	AMVN or AAPH	ABTS●		
Test Cond.	43°C, pH 7.4	RT		
Measurement	Abs: 234 nm	Abs: 734 nm		
Solubility	Lipophilic	Lipophilic		
Synergy effect	15%	13%		
Binary mixtures of BHT with BHA and TBHQ [BHT + BHA, 50 μL 1 mM + 50 μL1mM (in DCM/EtOH, 9:1 v/v)] *Celik et al. (2010)*	*Assay*	CUPRAC/CUPRAC-DCM	2ABTS	FRAP	Polymerizetion, deploymerization, and H-bonding interactions among AOXs themselves and solvent molecules
Inducer	Cu(II)–Nc	ABTS·	TPTZ–Fe^{3+}	
Test Cond.	RT, 30 min 4°C, 30 min	RT	37°C, pH 3.6	
Measurement	Abs: 450 nm	Abs: 734 nm	Abs: 595 nm	
Solubility	Hydro/lipophilic	Hydro/lipophilic	Hydro/lipophilic	
Synergy effect	100%	77%	N.D	
Binary mixtures of α-TOH with 5 FOHs (MY, Q, QG, QR, R, MO, K, KG) [α-TOH + MY, 1:1 α-TOH + Q, 1:1 α-TOH + K, 1:1] *Zhou et al. (2005)*	*Assay*	Lipid oxidation (homogeneous systems)	Lipid oxidation (heterogeneous systems)	FOHs partially regenerated α-TOH in micelles
Inducer	AAPH	AAPH		
Test Cond.	37°C, in tert-butyl alcohol/water (3:2)	37°C, pH 7.4, SDS or CTAB micelles		
Measurement	Abs: 235 nm	Abs: 235 nm		

(*Continued on next page*)

Table 1. (*Continued*)

Assayed mixtures [Identified synergy] *References*	Antioxidant capacity assays involved				Mode of action
	Solubility	Lipophilic	Lipophilic		
	Synergy effect	78%	91% (SDS)		
		48%	28% (CTAB)		
		33%	75% (SDS)		
			21% (CTAB)		
			29% (SDS)		
			29% (CTAB)		
Binary mixtures of CPL with AIL	*Assay*	TEAC	FRAP	DPPH	TBD
	Inducer	ABTS·	Fe^{3+}	DPPH·	
	Test Cond.	RT, dark, 16 h	50°C, 20 min, pH 6.6	RT, dark, 30 min	
	Measurement	Abs: 734 nm	Abs: 700 nm	Abs: 517 nm	
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic	
[CPL + AIL, 1:1] *Irondi et al. (2012)*	*Synergy effect*	10%	3%	4%	

Synergy effect (%) = 100% × (differences between the observed value and the theoretical value)/theoretical value, where the observed value is obtained experimentally, while the theoretical value is obtained as the summation of antioxidant capacities of individual antioxidants.

α-TOH = α-tocopherol, AA = ascorbic acid, AAPH = 2,2′-azobis(2-amidinopropane) dihydrochloride, ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, ACEPg = acetone crude extracts of *Potentilla fruticosa* leaves, AIBN = 2, 2′-azobisisobutyronitrile, AIL = *Azadarichta indica* leaf, AP = ascorbyl palmitate, AMVN = 2, 20-azobis(2,4-dimethylvaleronitrile), AR = artichoke, ASP = almond skin polyphenolics, ASP_M = ASP extracted with methanol, ASP_G = ASP extracted with gastrointestinal juice, ASDB = L-ascorbyl 2,6-dibutyrate, AV = p-anisidine value, BHT = butylated hydroxytoluene, BHA = butylated hydroxyanisole, BO = borututu, BODIPY 581/591 = 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)- 4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, BR = Briggs–Rauscher, C3A = caspase-3 activity, CA = caffeic acid, CAT = catalase, CAPE = caffeic acid phenethyl ester, CATE = (+)-catechin, CD = conjugated diene, CGA = chlorogenic acid, CHP = cuminehydroperoxide, CPL = *Carica papaya* leaf, CTAB = cetyl trimethylammonium bromide, CuZnSOD = Cu-Zn superoxide dismutase, DHE = dihydroethidium, DHR 123 = dihydrorhodamine 123, DHS = delipidized human serum, DPPH = 2,2-diphenyl-1-picrylhydrazyl, DMSO = dimethyl sulfoxide, DSC = differential scanning calorimetry, EA = ellagic acid, EC = epicatechin, EC_{50} = concentration of sample to scavenge the free radical to 50% of initial concentration, ECG = epicatechin gallate, EG = (-)-epicatechin gallate, EGb = ginkgo biloba extract, EGCG = (-)-epigallocatechin gallate, EGC = (-)-epigallocatechin, ESP = espresso, EPR = electron paramagnetic resonance, EO = *Emblica officinalis*, FA = ferulic acid, FeII(CN)6 = ferrocyanide, FRAP = ferric ion reducing antioxidant power, GA = gallic acid, GOH = green tea polyphenols, GPx = glutathione peroxidase, GR = glutathione reductase, GST = glutathione S-transferase, GSH = glutathione, GT = green tea, GTE = green tea extract, HYPE = hyperoside, LA = linoleic acid, LAME = linoleic acid methyl ester, LDL = low density lipoprotein, L-7-O-G = luteolin-7-O-glucoside, K = kaempferol, KAEM = kaempferide, KG = kaempferol glucoside, IC_{50} = half-maximal inhibitory concentration, IR = inhibition ratio, IW = insoluble wheat, IR = insoluble rye, IP = induction period, IO = insoluble oat, ISOR = isorhamnetin, MMA = methyl methacrylate, MAD = malondialdehyde, MG = methyl gallate, MMb = Metmyoglobin, MnSOD = Mn superoxide dismutase, MMP-2A = matrix metalloproteinase-2 activity, MO = morin, MeO-AMVN = 2,20-azobis(4-methoxy-2,4-dimethylvaleronitrile), MT = milk thistle, MY = myricetin, NADPH = nicotinamideadenine dinucleotide phosphate, NBT = nitroblue tetrazolium, N.D. = not detected, Nrf2 = nuclear factor E2-related factor 2, PC = phosphatidylcholine, Q = quercetin, QG = quercetin galactopyranoside, QR = quercetin rhamnopyranoside, Q value = value calculated as $Q = <T_{sample} - T_{control}>/T_{control}$, where T_{sample} and $T_{control}$ represent the time required to reach half of the maximum fluorescence, SDS = sodium dodecyl sulfate, SA = *Semecarpus anacardium* nut milk extract, SOD = superoxide dismutase, S = syrup, SU = sumac, OE = rosemary extract, ORAC = oxygen radical absorbance capacity, PCL = phosphatidylcholine liposome, PJ = pomegranate juice, PV = peroxide value, R = rutin, RES = resveratrol, RE = rosemary extract, ROE = rooibos extract, RPO = red palm oil, RW = red wine, RS = raspberry, RSA = radical-scavenging activity, RT = room temperature, TAP = total antioxidant performance, TBA = 2-thiobarbituric acid, TBD = to be determined, TEAC = trolox equivalent antioxidant capacity, T_{inhib} = inhibition time, TOH = tocopherol, TPC = total phenolic content, TPTZ = 2,4,6-tripyridyl-s-triazine, VLDL = very low density lipoprotein.

[a]Four fruits including apple (*Malus domestica*), raspberry (*Rubus strigosus*), blackberry (*Rubus fruticosus*), and grape (*Vitis labrusca*); four vegetables including broccoli (*Brassica oleracea*), onion (*Allium cepa*), tomato (*Solanum lycopersicu*), and white button mushroom (*Agaricus bisporus*) and purple cauliflower (*B. oleracea*), and three dry leguminous seeds including black beans (*Phaseolus vulgaris*), adzuki beans (*Vigna angularis*), and red kidney beans (*P. vulgaris*).

[b]Eleven flavonoids including peonidin-3-*O*-glucoside (P), cyanidin-3-*O*-glucoside (CY), delphinidin-3-*O*-glucoside (DP), malvidin-3-*O*-glucoside (MV), pelargonidin-3-*O*-glucoside (PG), catechin (CAT), epicatechin (ECAT), kaempferol (K), myricetin (MY), quercetin (Q), and quercetin-3-*β*-glucoside (Q-3-G).

[c]Ten flavonoids including taxifolin, (+)-catechin, luteolin, luteolin-7-*O*-glucoside, quercetin, quercetagetin, myricetin, isorhamnetin, galangin, and fisetin.

superoxide) scavenging abilities, but were antagonistic in the ORAC assay. Celik et al. (2010) also found that interactions between BHT and BHA were synergistic in the ABTS assay, but not in FRAP assay (determining the reducing power of AOX). The extent of synergy is also method sensitive. A typical example was provided by Celik et al. (2013), who studied beverage mixtures, and found that green tea (GT) associated mixtures showed the highest synergistic effect using the ESR assay, whereas espresso-related mixtures showed the highest synergy in the liposome oxidation assay.

Another concern associated with the chemical assays is the time chosen for completing reaction between the AOXs and the probes (i.e., Fe^{3+} probe for FRAP). Taking DDPH assay as an example, 20, 30, and 60 minutes of reaction time were used to determine the AOX synergy of binary mixtures of caffeic acid phenethyl ester (CAPE) and trolox (Bai et al., 2014), ternary extract mixtures of medical plants [artichoke (AR), borututu (BO), milk thistle (MT)] (Pereira et al., 2013, 2014), and binary mixture of RES with GA, CATE, or quercetin (Q), CA (Skroza et al., 2015), respectively. Increasing the reaction time from 30 to 60 minutes did not change the synergistic effect of RES and CA, but increased that of RE and CAT by 12% (Skroza et al., 2015). Another example being FRAP assay, diverse reaction lengths were chosen to determine the AOX capacity of binary mixtures of 11 flavonoids (15 minutes) (Hidalgo et al., 2010), RE with GA, CATE, Q, and CA (4, 8, and 20 minutes) (Skroza et al., 2015), CPL with AIL (20 minutes) (Irondi et al., 2012), and CATE with eight phenolics (30 minutes) (Wang et al., 2015a). The mixture of RE with CA had a 16% decrease in synergy effect, and the mixture of RE and CATE had an 80% increase when prolonging the reaction time from 8 to 20 minutes (Skroza et al., 2015). Reaction time should, thus, be carefully noted. Overall, there is a lack of consistency and standardization in the

synergy quantification methods (Perez-Jimenez and Saura-Calixto, 2005).

Composition of AOX

Chemical structure, concentration, and ratio of AOXs in the mixture can affect the synergy. For example, no AOX synergy was seen at various concentrations, except for mixing α-TOH (200 μg/g oil) and γ-TOH (200 μg/g oil) at 1:1 (Kamal-Eldin and Budilarto, 2014), α-TOH and CATE at 1:2 or 2:1 (Yin et al., 2012), α-TOH (1.0 μM) and lycopene (12.5 μM) at 1:1 (Shi et al., 2010), α-TOH and *Centella asiatica* extract at 3:2 (Thoo et al., 2013), as well as α-TOH and Q in micelles at 1:1 (Zhou et al., 2005). A variation in α-TOH associated synergies depends on the structural properties of individual AOX, solubility, reaction mechanism, and reaction medium (Zhou et al., 2015). For example, in SDS micelles (globular core-shell micelles in an aqueous solution), lipophilic Q bound to micellar surface by hydrogen-bonding and trapped a-tocopheroxyl radicals, regenerating α-TOH (Zhou et al., 2005). This resulted in synergy of Q and α-TOH. On the other hand, α-TOH could not synergize with EC because of the steric effect (Kadoma et al., 2006).

Solvent effect

Diverse solvents (e.g., water, aqueous buffers, ethanol, ethanol/water, acetone/water, methanol/water, chloroform, and acidic methanol/water) were used for sample extraction and reaction medium in different studies. Extraction solvent (methanol, acetone, chloroform) effects have been linked to the AOX capacity of the mixtures (Serrano et al., 2005). By means of CUPRAC, ABTS, and FRAP assays, different AOX capacities were seen in the binary mixture of BHT with BHA or GT, when varying solvent composition, including 100% ethanol (EtOH), 100% methanol (MeOH), 80% MeOH/20% H_2O, 50% MeOH/50% H_2O, and 90% dichloromethane (DCM)/10% EtOH. Extraction solvent may interact with reaction medium to affect the synergy of AOX mixtures. Serrano et al. (2005) revealed that BHT synergized with BHA in DCM/EtOH medium (nonpolar environments) in the CUPRAC and ABTS assays, but no synergy in FRAP assay (FRAP is nonresponsive in DCM medium). This nonresponsive phenomenon could be due to noncompatibility between the DCM and FRAP working solution, which was composed of aqueous TPTZ solution and ferric chloride in sodium acetate buffer (pH 3.6). Thus, these chemical assays determine AOX capacity of soluble proportion of samples, only if the extract solvent is compatible to reaction medium of the assays. In addition, Celik et al. (2015) found metal ion residues, obtained from the extraction of grain samples, promoted lipid oxidation, thus underestimated the synergy potential of samples in inhibiting lipid oxidation. So synergy inhibitors (e.g., metal ion) and promoters should be limited or avoided.

The reaction medium possibly influences the relative contribution of hydrogen abstraction and electron transfer of AOX on the synergy. Hydrogen bonding in polar solvents may induce dramatic changes in the H-atom donor activities of phenolic AOXs, consequently affecting the measured AOX capacity (Zhou et al., 2005). For example, the synergy of BHT and BHA was most likely due to depolymerization and intermolecular H-bonding reactions in a nonpolar environment (DCM/EtOH medium) and the easier accessibility of the phenolic–OH groups (Serrano et al., 2005). For a wide range of assayed mixtures, reaction medium used for synergy qualification can either be homogenous or heterogeneous. Homogenous media included solutions of free radicals (DPPH, ABTS, APPH), of linoleic acid methyl ester (LAME), and of BuOH/H_2O (Zhou et al., 2005). Heterogeneous media were soybean oil or canola emulsions (Dhi et al., 2007; Kamal-Eldin and Budilarto, 2014; Winkler-Moser et al., 2014), and artificial membranes (liposomes, micelles) in buffer solutions (Zhou et al., 2005; Becker et al., 2007; Liang et al., 2009; Yin et al., 2011). The performances of AOXs in homogeneous solution (a single phase) differ from that in heterogeneous (multiphase) systems, due to more complex solvation and interfacial phenomena affecting the interactions (Zhou et al., 2005; Becker et al., 2007). For example, Zhou et al. (2005) compared the possible synergy of binary mixtures of α-TOH with five flavonols (FOHs) in preventing linoleic acid from peroxidation in homogeneous solution (tBuOH/H_2O, 3:2) and in heterogeneous solutions [sodium dodecyl sulfate (SDS) and cetyl trimethylammonium bromide micelles]. In the homogeneous solution, hydrophilic FOHs trapped the initiating radicals to reduce the effective initiation, therefore synergizing with lipophilic α-TOH. In the heterogeneous solution, FOHs trapped the propagating lipid peroxyl radicals on the surface of the micelles, and reduced the α-tocopheroxyl radicals to regenerate α-TOH, synergizing with α-TOH (Zhou et al., 2005). Kamal-Eldin and Budilarto (2014) found that the difference in synergy between α-TOH and γ-TOH in canola oil and soybean oil emulsions was associated with the fatty acid composition of oils. Canola oil has 66.9, 17.7, and 6.2%, and soybean oil has 23.4, 53.1, and 6.7% oleic, linoleic, and linolenic acids, respectively. This difference in fatty acid composition as well as the assayed AOXs resulted in different packing of triacylglycerols and oxidation vulnerability. Similarly, Becker et al. (2007) investigated the possible synergy of binary mixture of α-TOH and Q in three lipid model systems, including bulk oil (high oleic sunflower oil), O/W-emulsion (methyl linoleate emulsion), and liposomal suspension [L-α-phosphatidylcholine (PC) liposome]. Q only synergized with α-TOH in methyl linoleate emulsions (metmyoglobin initiated oxidation) and in liposome (AAPH initiated oxidation), but not in the bulk oil system.

Regeneration hypothesis

In chemical systems, regeneration hypothesis refers to a synergy that occurs when individual AOXs in combination act in a regenerating manner, with a weaker one regenerating a stronger one (Peyrat-Maillard et al., 2003; Kiokias et al., 2008; Vijayalakshmi et al., 2014). AOX regeneration has been used to explain synergy of binary mixtures of chlorogenic acid (CGA) and β-carotene in the presence of t-BuOOH· (Vijayalakshmi et al., 2014), δ-TOH, and EC in the presence of PhCOO· (Kadoma et al., 2006), and a ternary mixture of EC, α-TOH, and AA in the presence of AAPH· (Dai et al., 2008). The chemical features of AOXs determined the occurrence of regeneration (Karabulut, 2010; Vijayalakshmi et al., 2014). For example,

AA was able to regenerate α-TOH and yielded a synergy with α-TOH, possibly via a synthesis reaction (LOO· + TOH → LOOH + TO· and AscH− + TO· → Asc·− + TOH) (Peyrat-Maillard et al., 2003). The δ-TOH, rather than α- and β-TOH, synergized with EC, due to the lower steric effect of δ-TOH on the regeneration between δ-TOH and EC (Karabulut, 2010; Decker et al., 2008). The synergy of α-TOH and β-carotene was due to β-carotene donating hydrogen to inhibit tocopheroxyl radicals from inducing propagation phase of lipid peroxidation (Decker et al., 2008). In some scenarios, regeneration reaction may not be adequate to explain the AOX synergy. The occurrence of regeneration (radical exchange between two AOXs) depends on the behaviors of AOX involved. For example, a synergy between β-carotene and α-TOH in butter oil triacylglycerols was based on the radical-trapping function of β-carotene, rather than hydrogen donation to α-TOH (Karabulut, 2010).

Synergy in food and biological systems

Compared with the above-mentioned chemical models, synergy studies in biological systems appear much less due to higher cost, greater difficulty in sample handling, and much complicated nature (Table 2). Established models of food and biological systems included food matrix (e.g., meat ball and bread) (Yang et al., 2011; Serpen et al., 2012; Karpińska-Tymoszczyk, 2014), biomaterials (e.g., human low density lipoprotein and human placental mitochondria) (Chen et al., 2007; Milczarek et al., 2010), living cells (e.g., cardiomyocytes H9c2 cells, human colon carcinoma HT-29, and SW-620 cells) (Santandreu et al., 2011; Wang et al., 2013), animals (e.g., Wistar and Albino rats) (Asdaq and Inamdar, 2010; Ajuwon et al., 2013), and humans (Bruno et al., 2006).

Food models

Focusing on rancidity-susceptible meat products, the use of synergy of AOXs formulated in the products at different concentrations and ratios to prolong the shelf life was studied, in combination with different packaging conditions (Karpińska-Tymoszczyk, 2014). In a study by Karpińska-Tymoszczyk (2014), lipophilic RE synergized with BHT in increasing oxidative stability (35%), which maintained the color and flavor of air packaged cooked turkey meatballs stored at −20°C up to 90 days. The lipid oxidation was determined using thiobarbituric acid-reactive substances (TBARS) and descriptive sensory evaluation. The presence of AOX synergy retarded metmyogobi (MMb) formation in turkey meatballs (Karpińska-Tymoszczyk, 2014). A study of Romano et al. (2009) emphasized the possible inhibition effects of the AOX synergy for antimicrobial application. RE (containing 30% carnosic acid, 16% carnosol, and 5% rosmarinic acid) was found to synergize with BHA in inhibiting the growth of two food-borne bacteria: *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive). In bread, Serpen et al. (2012) found the AOX synergy in bread from a grain mixture (rye with bran, oat, soybean, and maize) using ABTS and DDPH assays, but not from other multigrain combinations (binary, ternary, and quaternary). These studies initiated the use of AOX synergy for food applications. However, the mode of actions behind the synergies is hardly addressed.

Tissue culture

AOX synergy triggers the cellular AOX activities that are possibly relevant to AOX functions in vivo. Cultured cells used in synergy investigations included rat cardio-myocyte H9c2 cells, human spontaneously immortalized breast epithelial MCF-10A cells, human live HepG2 cells, human colon carcinoma HT-29 and SW-620 cells, and human breast cancer MCF-7 cells (Bruno, et al., 2006; Wang et al., 2012; Ajuwon et al., 2013; Sen et al., 2013; Asokkumar et al., 2014; Pereira et al., 2014).

Possible synergy of plant-based food extracts in protecting the H9c2 cells from exogenously exposed H_2O_2 was evaluated. A binary mixture of raspberry and adzuki bean extracts synergistically reduced H_2O_2-induced H9c2 cell death by 21%, cell apoptosis (reflected through caspase-3 activity) by 20%, and matrix metalloproteinase-2 activity by ∼10% (Wang et al., 2012). HepG2 cells were applied to determine possible synergy of three medicinal plants (AR, BO, and MT). A mixture containing hydrophilic extracts of 15% AR, 15% BO, 35% MT, and sweetener (sodium saccharin) synergistically increased anti-hepatocellular carcinoma activity by 22% (Pereira et al., 2014). Possible synergistic cytotoxicity of the mixture of RES and chemotherapy drug 5-fluorouracil (5-FU) toward cancerous cells (HT-29 and SW-620 cells) were evaluated (Santandreu et al., 2011). When applied to cell culture for 48 hours, RES (100 μM) synergized with 5-FU (10 μM) in promoting the cytotoxicity to cancer cells by inactivation/downregulation of redox-sensitive proteins. However, relative effectiveness of the mixture of RE and 5-FU on the two cancerous cells was different. In the case of HT-29 cells, the synergy effects of the mixture on inhibiting cellular lipid peroxidation, and increasing CAT, and glutathione peroxidase/superoxide dismutase activity ratio (GPx/COD) were 67, 45, and 19%, respectively. Using SW-620 cells, the synergy effects of the mixture on inhibiting cellular lipid peroxidation, and increasing CAT, and GPx/COD were 50, 55, and 23%, respectively. Thus, it appears that the extent of synergistic effect depends on the types of cell and AOX. Tissue culture-based evaluation suggests AOX synergies have several mechanisms of action, such as inhibition of oxidative enzymes responsible for the formation of reactive oxygen and nitrogen species (ROS/RNS), induction of expression of defense enzymes (SOD, CAT), scavenging ROS/RNS before they induce oxidative damages on biomolecules, modulating macromolecular interactions, and expression of genes associated with redox processes at physiological states (Santandreu et al., 2011; Wang et al., 2012; De Maria et al., 2013).

As discussed above, living cells better mimic the synergy behaviors at physiological states than chemical models. Methodologies for determining biomarkers associated with cell viability, AOX biomarkers, and redox prosurvival pathways have been established (Santandreu et al., 2011; Wang et al., 2012). However, cell-based evaluation hardly provides information on the metabolism of AOX synergy in vivo.

Table 2. Synergy representatives in food and biological systems (2005–2015).

Assayed combinations [Synergy, matrix] *Reference*		Antioxidant capacity assays involved		Mode of action
Food				
Binary mixtures of OE with BHT	*Assay*	Lipid oxidation		TBD
	Inducer	TBA		
	Test Cond.	Frozen sample stored for 90 days before thawing at 4°C for analysis, air or vacuum package		
	Measurement	Abs: 532 nm		
	Solubility	Lipophilic		
[OE + BHT, raw turkey meat balls]	*Synergy effect*	35% (air packaged)		
Karpińska-Tymoszczyk (2014)				
		26% (vacuums packed)		
Binary, ternary, quaternary, and quinary grain mixtures of breads	*Assay*	ABTS	DPPH	TBD
[wheat + oat + rye + soy + maize, bread]	*Inducer*	ABTS·	DPPH·	
Serpen et al. (2012)				
	Test Cond.	RT, dark	RT, dark	
	Measurement	Abs: 734 nm	Abs: 492 nm	
	Solubility	Hydrophilic	Hydrophilic	
	Synergy effect	11%	0.3%	
Biological materials				
Binary mixtures of CAPE with trolox	*Assay*	Lipid oxidation		TBD
[CAPE + Trolox (0.5 μM + 0.5 μM), microsomes]	*Inducer*	AA/Fe^{2+}, CHP, CCl_4/$NADPH^-$		
Bai et al. (2014)				
	Test Cond.	37°C, pH 7.4, 15 min, rat liver microsomes		
	Measurement	Abs: 535 nm Fluorescence, ex/em: 553/515 nm		
	Solubility	Hydrophilic		
	Synergy effect	47% (AA/Fe^{2+}) 266% (CHP) 25% (CCl4/$NADPH^-$)		
Binary mixtures of α-TOH with AA, β-carotene, and uric acid	*Assay*	Lipid oxidation		TBD
	Inducer	MeO-AMVN		
	Test Cond.	37°C, pH 7.4, 210 min (5 min interval), DHS with PCL liposomes		
	Measurement	Fluorescence, ex/em: 500/520 nm		
	Solubility	Hydro/lipophilic		
[α-TOH + AA (5 μM + 5 μM)	*Synergy effect*	84%		
β-carotene + AA (0.5 μM + 2.5 μM)		140%		
β-carotene + α-TOH (0.5 μM + 2.5 μM) α-TOH + uric acid (10 μM + 5.0 μM), liposomes]		100%		
Yeum et al. (2009)				
		122%		
Cell lines				
Binary mixture of CAPE and trolox	*Assay*	Cellular oxidative status	Redox related biomarkers	Multitargeting redox sensitive biomarkers
	Inducer	20 Gy radiation	20 Gy radiation	
	Test Cond.	37°C, 30 min95°C, 60 min	Incubated with antibodies	
		RT, 30 min, pH 8 L929 cells		
	Measurement	Fluorescence: Ex/Em: 553/515 nm; 420/350 nm	Western blotting	
	Solubility	Hydrophilic	Hydrophilic	

(*Continued on next page*)

Table 2. (*Continued*)

Assayed combinations [Synergy, matrix] *Reference*		Antioxidant capacity assays involved		Mode of action
[CAPE + Trolox (0.5 μM + 0.5 μM), cells] *Bai et al. (2014)*	*Synergy effect*	41% (ROS level)	58% (Nrf2)	
		38% (TBARS content)	39% (GR)	
		5% (GSH)	62% (Gpx)	
		25% (GSSG)		
		14% (GSH/GSSG)		
Ternary mixtures of AR with BO and MT [AR + BO + MT (15% + 15% + 35%), cells] *Pereira et al. (2014)*	Assay	Antiproliferation		TBD
	Inducer	N/A		
	Test Cond.	Human HepG2 cells		
	Target	SRB assay		
	STD	IC_{50}		
	Measurement	Cell		
	Solubility	Hydrophilic		
	Synergy effect	22%		
Binary mixtures of polydatinn with RES	*Assay*	Biochemical assays		TBD
	Inducer	ISBn		
	Test Cond.	Human colorectal Caco-2 cells		
	Measurement	Confocal analysis		
	Solubility	Lipophilic		
[polydatin + RES (3:1), cells] *De Maria et al. (2013)*	*Synergy effect*	5% (TBARS)		
		14% (NO^{2-})		
Binary mixtures of RES with 5-FU	*Assay*	Antiproliferation		Multitargeting redox sensitive biomarkers
	Inducer	H_2O_2		
	Test Cond.	1 h pretreatment, H9c2 cells		
	Measurement	SRB; caspase-3/7 fluorescence assay; gelatin zymography		
	Solubility	Hydrophilic		
[RS + AD (1:1), cells] *Wang et al. (2012)*	*Synergy effect*	21% (cell viability) 20% (C3A)		
		10% (MMP-2A)		
Binary mixtures of AA with ASP-M and ASP-G	*Assay*	Quinone reductase activity		TBD
	Inducer	N.A.		
	Test Cond.	48 h incubation, murine hepatoma Hepa1c1c7 cells		
	Measurement	Abs: 570 nm		
	Solubility	Hydrophilic		
[ASP-M +AA, cells] *Chen and Blumberg (2008)*	*Synergy effect*	18% (1 μmol/L)		
		13% (10 μmol/L)		
Binary mixtures of RES with 5-FU	*Assay*	Antiproliferation	Antiproliferation	Multitargeting redox sensitive biomarkers
	Inducer	tert-BuOOH	tert-BuOOH	
	Test Cond.	48 h treatment, HT-29 cells	48 h treatment, SW-620 cells	
	Measurement	MTT Western blot	MTT Western blot	
	Solubility	Hydrophilic	Hydrophilic	
[5FU + RES (10 mM + 100 μM), cells] *Santandreu et al. (2011)*	*Synergy effect*	29% (ROS levels)	10% (ROS levels)	
		50% (lipid peroxidation)	67% (lipid peroxidation)	
		55% (CAT)	45% (CAT)	
		23% (Gpx/COD)	19% (Gpx/COD)	
		46% (phospho-AKT)	38% (phospho-AKT)	
		33% (phospho-STAT3)	57% (phospho-AKT)	
Binary mixtures of sumac with RS [Sumac + RS (1:1), cells] *Wang et al. (2015a)*	*Assay*	Selective antiproliferation	Selective antiproliferation	TBD
	Inducer	N/A	N/A	
	Test Cond.	MCF-10A and MCF-7	4D/WT and D/V-Sr cells	
	Measurement	SRB	SRB	
	Solubility	Hydrophilic	Hydrophilic	

(*Continued on next page*)

Table 2. (*Continued*)

Assayed combinations [Synergy, matrix] *Reference*		Antioxidant capacity assays involved		Mode of action		
	Synergy effectCell viability	2% (MCF-10A)	2% (4D/WT)			
		14% (MCF-7)	9% (D/V-Sr)			
Animals						
Binary mixture of ROE and PROE	*Assay*	FRAP	TEAC	ORAC	Oxidative status	Multitargeting redox sensitive biomarkers
	Inducer	TPTZ–Fe^{3+}	ABTS•	AAPH	tert-BuOOH	
	Test Cond.	37°C, 30 min pH 3.6, in plasma and liver HTH	RT, pH 7.4, in plasma and liver HTH	pH 7.4, in plasma and liver HTH	In plasma and liver	
	Measurement	Abs: 593 nm	Abs: 734 nm	Fluorescence ex/em: 485/538nm	HPLC, Abs: 234 nm biochemical	
	Solubility	Hydrophilic	Hydrophilic	Hydrophilic	Hydrophilic	
[RTE + RPOE (2% w/v + 7 g/kg diet), Wistar rats] *Ajuwon et al. (2013)*	*Synergy effect*		CD MDA		CD MDA 4% 0.3%	
	plasma	17%	12% 4%	15%	3% 15%	
	liver	4%	4% 10%	6%		
Binary mixtures of GH with HCTZ and CAP	*Assay*	Biochemical changes and antioxidant profile in serum	Biochemical changes and antioxidant profile in tissue HTH	Multitargeting redox sensitive biomarkers		
	Inducer	GH	GH			
	Test Cond.	30 days, GH pretreatment of albino rats	30 days, GH pretreatment of albino rats			
	Measurement	Biochemistry	Biochemistry			
	Solubility	Hydrophilic	Hydrophilic			
[GH + HCTZ, albino rats] *Asdaq and Inamdar (2010)*	*Synergy effect*	4%/13% (MDA)	9%/16% (SOD)			
		11%/12% (GSHPx)	13%/29% (CAT)			
		25%/25% (LDH)	18%/17%(LDH)			
		20%/32% (CK-MB)	17%/24% (CK-MB)			
			22%/13% (TBARS)			

Synergy effect (%) = 100% × (difference between observed value and theoretical value)/theoretical value, where the observed value is obtained experimentally, while the theoretical value is obtained as the summation of antioxidant capacities of individual antioxidants.

Cell lines include: 4D/WT = rat colonic epithelial cells, D/V-Src = transformed colonic epithelial cells, MCF-10A = human spontaneously immortalized breast epithelial cells, MCF-7 = human breast cancer cells, HT-29 = human colon carcinoma cells, SW-620 = human colon carcinoma cells.

5-FU = 5-fluorouracil, α-TOH = α-tocopherol, AA = ascorbic acid, AAPH = 2,2′-azobis(2-amidinopropane) dihydrochloride, ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, AOX = antioxidant, CAP = captopril, CAT = catalase, CD = conjugated dienes, CK-MB = creatine phosphokinase isoenzyme, GSHPx = glutathione peroxidise enzyme activity, HCTZ = hydrochlorothiazide, HTH = heart tissue homogenate, ISBn = hydroxystilbenes, LDH = lactate dehydrogenase, MDA = malondialdehyde, RES = resveratrol, RS = raspberry, SOD = superoxide dismutase, STD = standard, SU = sumac, TBARS = thiobarbituric acid reactive species.

Animal and clinical models

Animal and clinical models perfectly mimic the pathogenicity of OS-associated disorders, which involve a complexity of physiological, immunological, environmental, and genetic phenomena. The advantage of animal and clinical models over living cell models is its ability to reveal bioavailability and metabolism of AOXs in synergy.

In an animal feeding experiment, aqueous rooibos extract (ARE) (*Aspalathus linearis*) synergized with red palm oil (RPO) *(Elaeis guineensis*) in preventing OS formation and ameliorating hepatotoxicity induced by tert-butyl hydroperoxide (*t*-BHP) in male Wistar rats. Lipid peroxidation and modulation of endogenous AOX enzymes and glutathione status were improved through this synergy (Ajuwon et al., 2013). Asdaq and Inamdar (2010) reported that oral administration of garlic homogenate (GH) (125, 250 mg/kg) along with hydrochlorothiazide (HCTZ) and captopril (CAP) synergistically elevated the activities of endogenous AOX enzymes (e.g., SOD and CAT) in female Wistar albino rats. Asokkumar et al. (2014) found that GA (50 mg/kg) synergized with famotidine (10 mg/kg) in protecting the gastric mucosa of rat significantly. This was evidenced by increases in the activities of endogenous AOX enzymes and a decrease in lipid peroxidation in rat stomach. In a human study, AA synergized with α-TOH in decreasing the rate of α-TOH disappearance in the plasma of smokers. This is the only in vivo example demonstrating the AOX regeneration theory as proposed by Bruno et al. (2006). There is yet no optimal model to study the mechanism and determine the effectiveness of the AOX synergy in vivo.

Correlations between synergies in chemical and biological systems

Diverse chemical assays have been developed with an aim to mimic the actual situation in human body. Chemical models can be realistic assumptions only if a positive correlation

between chemical and biological models is established. It has been claimed that hydrogen atom transfer (HAT) and single electron transfer (SET) are relevant mechanisms for dietary AOX activity in the human body (Ou et al., 2002; Huang et al., 2005). ORAC assay is among the commonly used assays to reflect the former, while FRAP and DDPH are among the commonly used assays to reflect the latter. It has been hypothesized that a combination of AOXs that can contribute to both HAT and SET systems may result in a more powerful AOX response at physiological levels (Wang et al., 2011). A synergistic response in chemical systems (DDPH, FRAP, and ORAC assays) produced by the mixture of raspberry and adzuki bean extracts was also seen in preventing H9c2 cells from H_2O_2-induced damage (Wang et al., 2012). The synergy between Cape and trolox has been confirmed in both chemical systems using DPPH, hydroxyl, and superoxide anion, and biological systems of lipid oxidation in rat liver microsomes and oxidative status and biomarker expression in L929 cells (Bai et al., 2014). The synergies among three medicinal plants (AR, BO, and MT) were seen in radical scavenging activity, reducing power, and lipid peroxidation inhibition (chemical systems), and also in antihepatocellular carcinoma activity of HepG2 human cells (Pereira et al., 2014).

However, the synergistic AOX activities in chemical systems, revealed by the "test-tube" assays, may not correlate with biological activities. Supporting evidence was given by Wang et al. (2013), who observed that extract pairs (e.g., raspberry and adzuki bean) showed synergistic AOX activities in FRAP, DPPH (SET mechanism), and ORAC (HAT mechanism) assays, but not in antiproliferative effects (APE) against MCF-7 cancerous cells. Extract pairs (e.g., onion and grape) that showed synergy upon APE against MCF-7 cells did not exhibit any AOX synergy in chemical assays (Wang et al., 2012). Thus, AOX synergy estimations from chemical models may mislead the current synergy research as they are not synonymous with the synergy in biological systems.

Conclusions

A variety of studies proved the occurrence of the synergistic interactions in mixtures of pure AOX compounds and extracts in diverse chemical (most cases) and/or biological systems. These AOX synergies showed great variability due to the characteristics of the AOX constituents (structure, stability, concentration, and ratio) and estimation assay used. Much of the synergy estimation has been made in the chemical systems, which may not be relevant to the actual situation in food and biological systems. There is a lack of robust biological methods to quantify the synergy and their mechanisms. Substantial efforts are needed to correlate the synergy in chemical systems with that in biological systems. The following points should be considered in the future: (1) To improve the current models for synergy estimation. The proper environment and concentration of AOXs should mimic biological and food systems as much as possible. (2) To develop and validate food-based models for characterizing synergies involving food fortification and preservation. (3) To clinically confirm the AOX synergies in vivo, considering the optimal concentrations associated with bioavailability.

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