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THE SCAVENGING CAPACITY AND SYNERGISTIC EFFECT OF SOME NATURAL ANTIOXIDANTS MIXTURES

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ABSTRACT: ***The biological activity of the natural antioxidant vitamin C can be enhanced by the presence of some other active natural antioxidants in mixtures such as gallic acid and tannic acid. Since many of these natural antioxidants are consumed sometimes together in human foods, where the potency for synergistic interactions is high in the human diet. The current study was conducted to determine what concentrations and combinations of antioxidants among vitamin C, gallic acid and tannic acid were capable to produce the highest synergistic antioxidant effects. Scavenging capacities of solutions of the selected compounds alone and in different combinations were measured using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) method.***

Different levels of each antioxidant in mixtures were used. A comparison of the scavenging capacity (antioxidant activity) of these combinations to antioxidants arithmetic sum of the individual antioxidants scavenging capacity was used to calculate the synergistic effects (SEs) between the used antioxidants.

The results showed that the binary mixture combining vitamin C (5.0 µM) and tannic acid (5.0 µM) had the highest SEs. Also the results obviously showed that the antioxidant property of this combination was substantially superior to the sum of the individual antioxidant effects, and these interactions can enhance the antioxidant effectiveness of these natural antioxidants. The results could guide in the formulation and development of functional food products that have high antioxidant potency.

Key words: ***Antioxidant; vitamin C; DPPH•; scavenging capacity; synergistic effect***

INTRODUCTION

Antioxidants are considered important to be an nutrceuticals on account of many health benefits (Droge, 2002; Lee *et al.,* 2004; Valko *et al.,* 2007). Phenolic compounds like gallic acid, tannic acid, and vitamin E,… as well as vitamin C etc. are existed naturally in different plants and fruits. These compounds possess the ability to reduce oxidative damage, that are believed to cause many diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases

and ageing (Lee *et al.,* 2000; Middleton *et al.,* 2000; Pietta *et al.,* 1998). Recently, the ability of phenolic substances including flavonoids and phenolic acids to act as antioxidants have been extensively investigated (Rice-Evans *et al.,* 1996). Natural antioxidants exist in nature in combination, and a combination of different antioxidants might act additively and even synergistically (Fuhrman *et al.,* 2000). The radical-scavenging capacity was determined by the DPPH$^{\bullet}$ assay which is a rapid, easy and inexpensive way. DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) is a stable radical of organic nitrogen, characterized by a typical deep purple color and a maximum absorbance in the range of 515–520 nm (Eklund *et al.,* 2005). Scavenging of DPPH$^{\bullet}$ radical is the basis of the popular DPPH$^{\bullet}$ antioxidant assay (Alma *et al.,* 2003; Karioti, *et al.,* 2004; Kordali *et al.,* 2005). The DPPH$^{\bullet}$ method is technically simple and needs only a UV– VIS spectrophotometer to perform in the presence of a hydrogen/electron donor (free radical scavenging antioxidant). When the absorption intensity is decreased, and the radical solution is discolored according to the number of electrons captured (Markowicz Bastos *et al.,* 2007). The DPPH$^{\bullet}$ method was first reported by Blois (1958), who observed the reduction of the DPPH$^{\bullet}$ radical by the thiol-containing amino acid cysteine and other active compounds. Afterward, Williams *et al.,* (1995) revised the original method and the DPPH$^{\bullet}$ radical scavenging test became a reference point to evaluate the *in vitro* antioxidant capacity (Gil *et al.,* 2000). The purpose of this study was to compare the antioxidant activity of vitamin C, gallic acid and tannic acid at different concentrations, either alone or in combinations as well as the synergistic effects of these combinations, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical scavenging capacity assay.

MATERIALS AND METHODS

1. Materials

1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical and absolute extra ethanol were obtained from Sigma–Aldrich (U.S.A). All natural antioxidants (gallic acid, tannic acid and vitamin C) were used in pure form and purchased from Sigma-Aldrich. All reagents used in this study were of analytical grade.

2. Antioxidant solutions

The composition and concentration of individual antioxidant solutions were as follows:

a) - The individual antioxidant solution concentrations of vitamin C were (1,2,5,10 and 20 µM).

b) - The individual antioxidant solution concentrations of tannic acid were (1,2,5,10 and 20 µM).

c) - The individual antioxidant solution concentrations of gallic acid were (1,2,5,10 and 20 µM).

The composition and concentration of the binary and tertiary antioxidant mixture solutions are shown in table 1. Stock solutions were prepared in ethanol and stored at 0 °C in a tightly closed dark brown glass bottles. All procedures were performed under dim light and all stock solutions were stirred at room temperature for 10 min before further use.

3. DPPH• free radical-scavenging capacity assay

The radical-scavenging capacity was determined by The DPPH• assay to evaluate the antioxidant activity. To measure the scavenging capacity of an individual antioxidant or a mixture of antioxidants. The antioxidant(s) was subjected to preincubation at room temperature for 5 min in the dark. The required amount of antioxidant 2 ml was pipetted into a cuvette containing 1mL of DPPH• in ethanol (0.3 mmol/L, stored at 0 C°) at room temperature to start the reaction, *Mensor, et al.,*(2001). The mixtures were shaken quickly, placed into the cell holder and the absorbance was measured at 540 nm with a UV spectrophotometer (UV-Visible Recording Spectrophotometer).

Each cuvette was removed from the spectrophotometer and incubated at room temperature. The cuvettes were covered with an opaque container to make sure that they were not exposed to light. Absorbance at 540 nm was measured every 10 min over a 60 min time period (Fuhrman *et al.,* 2000).

4. Calculation of synergistic effects (SEs) of antioxidant mixtures

The experimental scavenging capacity (ESC) of antioxidant mixtures was calculated using the following equation:

$$\%ESC = 100 - ((Abs_{control} - Abs_{sample}) \times 100 / Abs_{control})).$$

Where: Abs_{sample} is the absorbance value of the sample (2.0 ml antioxidant(s) solution plus DPPH• solution 0.3 mM in ethanol) at each time interval, absolute ethanol was used instead of the sample solution as blank. $Abs_{control}$ is the absorbance value of control (DPPH• solution 0.3 mM in ethanol). The theoretical scavenging capacity (TSC) is the sum of the scavenging capacities of each antioxidant, calculated using the individual scavenging capacity in the following equation (Fuhrman *et al.,* 2000).

$$\%TSC = 100 - [(100 - ESC_{A1}) \times (100 - ESC_{A2}/100) \times (100 - ESC_{A3}/100) \times (100 - ESC_{A4}/100)]$$

Where: ESC_{A1}- ESC_{A4} represents the percentage ESC of the individual antioxidant.

A modified version of $\%TSC = 100 - [(100 - ESC_{A1}) \times (100 - ESC_{A2}/100) \times (100 - ESC_{A3}/100)]$

The synergistic effects (SEs) of the antioxidants combinations were based on the ratios of the experimental ESCs and the theoretical TSCs, which were calculated using the following equation (Fuhrman *et al.,* 2000).

SE = ESC/TSC,

Where: synergism was accomplished when SE was greater than 1(SE >1).

Table 1: Composition and concentration (μM) of binary and tertiary antioxidant mixture solutions.

Code	Vitamin C	Tannic acid	Gallic acid
Mix1	1.0	1.0	
Mix2	1.0		1.0
Mix3		1.0	1.0
Mix4	2.0	2.0	
Mix5	2.0		2.0
Mix6		2.0	2.0
Mix7	5.0	5.0	
Mix8	5.0		5.0
Mix9		5.0	5.0
Mix10	10.0	10.0	
Mix11	10.0		10.0
Mix12		10.0	10.0
Mix13	1.0	1.0	1.0
Mix14	2.0	2.0	2.0
Mix15	5.0	5.0	5.0
Mix16	10.0	10.0	10.0
Mix17	2.0	2.0	1.0
Mix18	2.0	1.0	2.0
Mix19	1.0	2.0	2.0
Mix20	5.0	5.0	10.0
Mix21	10.0	5.0	5.0
Mix22	5.0	10.0	5.0
Mix23	2.0	5.0	10.0
Mix24	2.0	10.0	5.0
Mix25	5.0	2.0	10.0
Mix26	5.0	10.0	2.0
Mix27	10.0	5.0	2.0
Mix28	10.0	2.0	5.0
Mix29	20.0	10.0	5.0
Mix30	20.0	5.0	10.0
Mix31	10.0	20.0	5.0
Mix32	10.0	5.0	20.0
Mix33	5.0	10.0	20.0
Mix34	5.0	20.0	10.0
Mix35	20.0	10.0	10.0
Mix36	10.0	20.0	10.0
Mix37	10.0	10.0	20.0

5. Statistical analysis

All the experiments were performed at least in triplicate (Snetecor and Cochran ,1967) using Microsoft Excel 2003 for comparison between (%ESC and %TSC).

RESULTS AND DISCUSSIONS

1. Antioxidant activity of single compounds

The changes in scavenging capacity of single antioxidant measured with the DPPH$^{\bullet}$ free radical is shown in Fig. 1 a, b and c. Where five levels of the concentrations (1,2,5,10 and 20 µM) of each individual antioxidant were used.

Tannic acid was a very efficient antioxidant, showing a large increase in scavenging capacity at all concentrations, where the single tannic acid at a concentration of 20.0 µM reached maximum experimental scavenging capacity (97.15 %) after 10 min only. The experimental scavenging capacities for the concentrations 5.0 and 10.0 µM showed 92.9 and 96.09 % respectively for % ESC (fig. 1c). In all cases the scavenging capacity had not strong increases after the first 10 min of the incubation period.

Studying the experimental scavenging capacity of vitamin C at the same concentrations (1,2,5,10 and 20 µM) revealed , an independent character, since the highest vitamin C concentration (20.0 µM) did not produce the highest scavenging capacity (% ESC = 65.41 %) (Fig. 1a). Low vitamin C concentration (2.0µM) was used to keep the scavenging capacity at reasonable levels (% ESC = 68.56 %). On the other hand, vitamin C 2.0 and 10.0 µM had insignificant difference, so their results were more better than concentrations 1.0, 5.0 and 20.0 µM of vitamin C. Similar results were reported by Liu, *et al*, 2008, where the reaction of vitamin C with DPPH$^{\bullet}$ was completed before 1 min from the starting. At tannic acid concentrations 1,2 µM, experimental scavenging capacity were 70.42 and 79.96 % respectively. The reaction of gallic acid with DPPH$^{\bullet}$ was similar to that with tannic acid in which the reaction was almost completed after 10 min and revealed dose dependence (fig. 1 b and c). In both cases, the highest antioxidant level produced the highest scavenging capacity values.

2. SE: binary-antioxidant mixture

Further studies were achieved to investigate the experimental scavenging capacity of combination of gallic acid and other antioxidants. Thus, among twelve possible combinations containing two antioxidants, only one combination (Mix. 7, Table 2), which contained vitamin C (5.0 µM) and tannic acid (5.0 µM) had the highest significant synergistic effect SE (SE > 1). Under these conditions, it is possible to say that tannic acid is regenerate vitamin C in Mix.7 (Fig. 2). At any case of binary mixture containing tannic acid at the same conditions, the experimental scavenging capacity was higher than the

others. Tannic acid was a very efficient antioxidant, showing a large increase in scavenging capacity in a dose dependent effect. That, show explaining the important of tannic acid and it's strongly scavenging activity against DPPH• free radical. The synergistic effect of tannic acid with vitamin C and gallic acid was clearly observed among the binary mixtures at the same conditions. While gallic acid with vitamin C in binary mixtures showed lower effect than tannic acid with vitamin C in binary mixtures at the same conditions (Table 2).

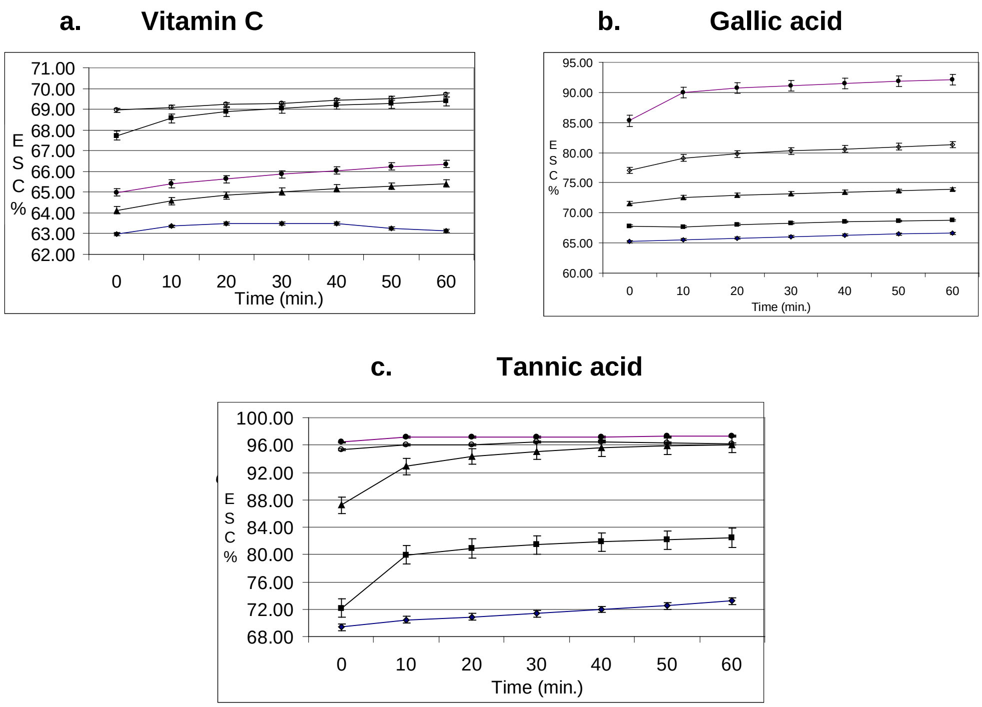


Fig. 1. Experimental scavenging capacity (ESC) kinetic curves of DPPH• free radicals by single antioxidants at various concentrations. Data represent means ±S.E. for n = 3. For the three compounds (a) V.C=Vitamin C; (b) G.A=Gallic acid; (c) T.A=Tannic acid by concentrations of (●, 20.0µM; ○,10.0µM; ▲,5.0µM; ■,2.0µM and ♦,1.0µM)

Table 2: Experimental scavenging capacity percentages (% ESC), theoretical scavenging capacity percentages (% TSC) and synergistic effect (SE) for binary antioxidant mixtures at 30 and 60 minutes

Code	30 min.			60 min.		
	%ESC	%TSC	SE	%ESC	%TSC	SE
Mix 1	74.67	89.55	0.83	75.63	90.11	0.84
Mix 2	69.22	75.72	0.91	69.39	75.45	0.92
Mix 3	73.43	90.29	0.81	74.35	91.04	0.82
Mix 4	76.24	94.25	0.81	77.81	94.62	0.82
Mix 5	67.37	90.19	0.75	67.78	90.44	0.75
Mix 6	81.46	94.11	0.87	82.68	94.51	0.87
Mix 7	95.15	93.50	1.02	95.22	93.92	1.01
Mix 8	69.64	90.63	0.77	70.88	90.98	0.78
Mix 9	92.90	98.68	0.94	92.90	98.98	0.94
Mix 10	92.50	98.78	0.94	92.54	98.72	0.94
Mix 11	79.57	93.26	0.85	80.70	93.71	0.86
Mix 12	91.55	99.30	0.92	91.48	99.29	0.92

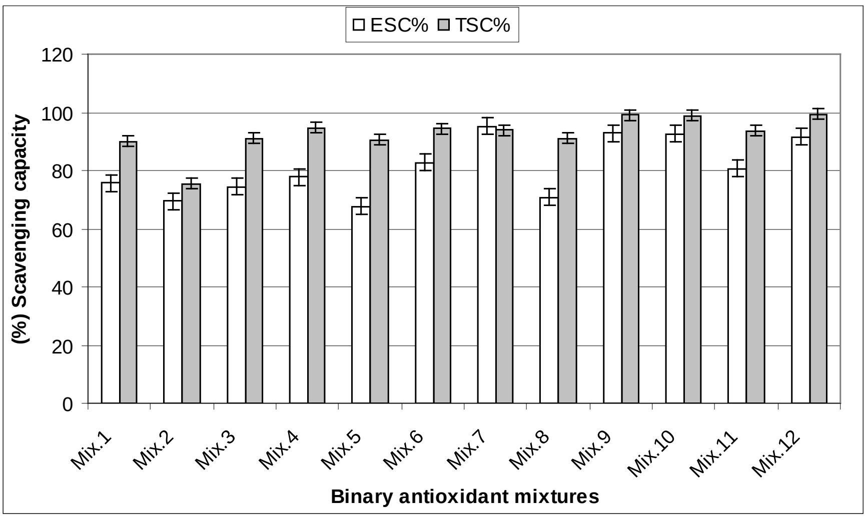
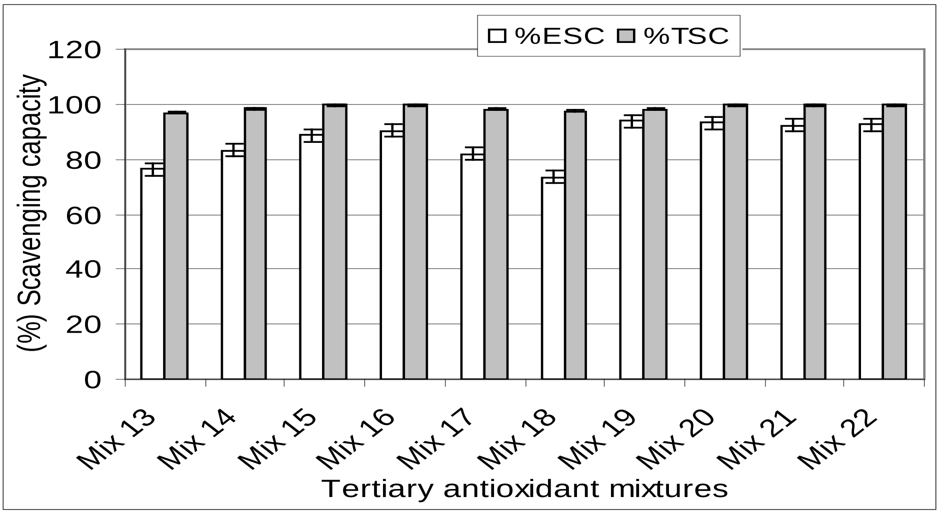


Fig. 2. Comparison of observed experimental scavenging capacity (□: ESC) and theoretical scavenging capacity (■: TSC) of antioxidant in binary-antioxidant mixtures at 60 min. Values are means ± S.E.



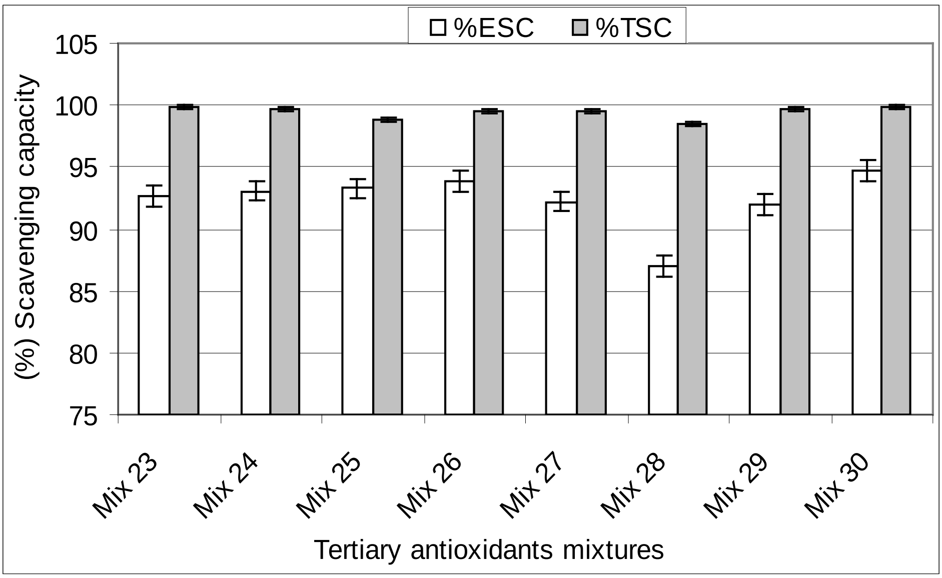


Fig. 3. Comparison of observed experimental scavenging capacity (□: ESC) and theoretical scavenging capacity (■: TSC) of antioxidant in tertiary-antioxidant mixtures at 60 min. Values are means ± S.E.

As shown in figure 2 only antioxidant mixtures Mix.7 had ESC/TSC ratios greater than 1. The mixture of tannic acid and vitamin C (Mix.7) showed a significant synergistic effect and SE > 1.01. When vitamin C was omitted from Mix.7 and replaced with gallic acid (Mix 9), the SE was decreased to 0.94. On the other hand, the mixture of tannic acid and vitamin C (Mix.10) which has the proper antioxidant composition, (tannic acid 10.0µmol/L and vitamin C 10.0µmol/L) produced SE ratio just like that of (Mix 9) i.e 0.94

3. SE: combination effects of three-antioxidant mixture

As shown in Table 3 and Fig. 3 there was not any tertiary antioxidant mixtures had ESC/TSC ratios greater than 1. The mixture of tannic acid, gallic acid and vitamin C (Mix.19) showed a significant synergistic effect and SE = 0.96. When vitamin C was omitted from Mix.19 as case of Mix.6 (Fig. 2) the SE decreased to 0.87.

Even more, the antioxidant mixture Mix.30, which has the proper antioxidant composition, produced SE ratio less than 1. The lack of agreement of such results in the present work with other studies might be due to different factors,such as that the present model system utilized for the free radical DPPH• that reacts with different antioxidants at different rates (Huang *et al.,* 2005) or that.Vitamin C reacts immediately with DPPH•, which would indicate that vitamin C reacted predominately with DPPH• and not with the antioxidant free radicals in some cases. The reaction mixture solvent in this study was ethanol. Sato *et al.* (1990) suggested that water-soluble chain radicals, such as vitamin C, function as a primary defense against aqueous radicals. Although Mix.37 (Fig. 4) had the highest concentrations of antioxidant mixtures, produced similar value (SE=0.93) at the same condition to that of Mix.10 (SE = 0.94) which did not contain gallic acid. Comparison of tertiary mixtures Mix. 17, Mix. 18 and Mix. 20 to binary mixtures which had omitted one antioxidant at the same conditions, showed that the results of these synergistic effects were almost-similar and had not strong differences (Table 2 and Table 3).

There are many factors that might contribute to SEs of mixed antioxidants in a biological system. The concentration and combination ratio of mixed antioxidants are the important factors. It has been shown that specific concentrations and antioxidants combinations in mixtures were more effective than the corresponding single antioxidants in the scavenging of DPPH•. In this study a significant SE was produced only when tannic acid was present in a mixture. For the mixtures of two-antioxidants, tannic acid with vitamin C, showed the highly SEs. For the mixtures of three-antioxidants any of them did not show synergism. The synergistic effect appeared to be based in part on the reducing potential of the various antioxidants and their ability to convert the antioxidant free radicals to their native form. The results

indicate that an optimum combination of antioxidants may play a significant role in enhancement of the oxidative status of biological systems.

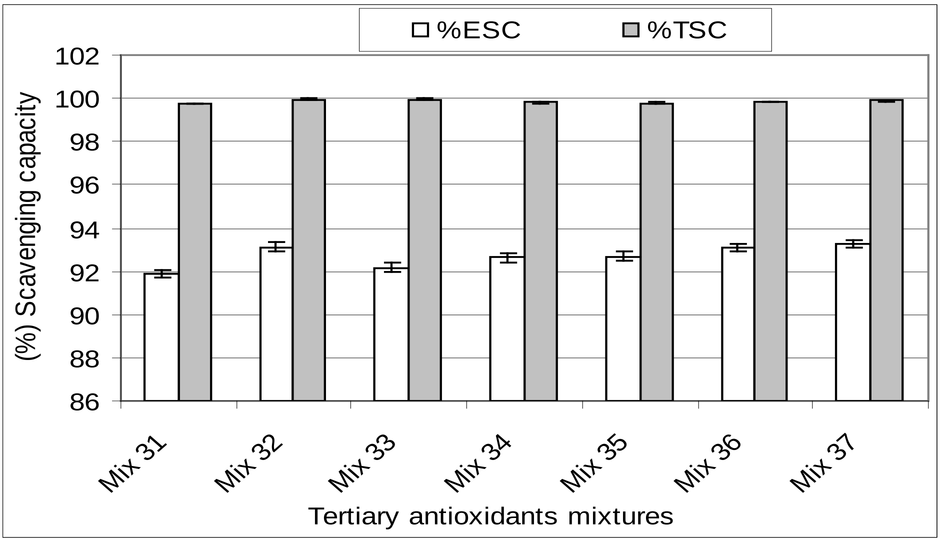


Fig. 4: Comparison of observed experimental scavenging capacity (□: ESC) and theoretical scavenging capacity (■: TSC) of antioxidant in tertiary-antioxidant mixtures at 60 min. Values are means ± S.E.

Table 3: Experimental scavenging capacity percentages (% ESC), theoretical scavenging capacity percentages (% TSC) and synergistic effect (SE) for tertiary antioxidant mixtures at 30 and 60 minutes

Code	30 min.			60 min.		
	%ESC	%TSC	SE	%ESC	%TSC	SE
Mix 13	75.30	96.46	0.78	76.29	96.70	0.79
Mix 14	81.57	98.18	0.83	83.31	98.32	0.85
Mix 15	88.46	99.54	0.89	88.62	99.65	0.89
Mix 16	90.54	99.76	0.91	90.46	99.76	0.91
Mix 17	80.56	98.05	0.82	81.93	98.20	0.83
Mix 18	72.51	97.16	0.75	73.61	97.44	0.76
Mix 19	93.86	97.85	0.96	93.86	97.98	0.96
Mix 20	93.17	99.66	0.93	93.13	99.75	0.93
Mix 21	92.14	99.55	0.93	92.18	99.66	0.92
Mix 22	92.62	99.67	0.93	92.62	99.66	0.93
Mix 23	92.78	99.70	0.93	92.70	99.78	0.93
Mix 24	93.07	99.70	0.93	93.07	99.71	0.93
Mix 25	93.27	98.72	0.94	93.27	98.86	0.94
Mix 26	93.86	99.60	0.94	93.86	99.59	0.94
Mix 27	92.09	99.47	0.93	92.20	99.59	0.93
Mix 28	85.57	98.30	0.87	87.04	98.46	0.88
Mix 29	91.77	99.71	0.92	92.00	99.70	0.92
Mix 30	94.76	99.70	0.95	94.68	99.78	0.95
Mix 31	91.86	99.74	0.92	91.90	99.76	0.92
Mix 32	93.17	99.95	0.93	93.13	99.96	0.93
Mix 33	92.14	99.96	0.92	92.18	99.96	0.92
Mix 34	92.62	99.81	0.93	92.62	99.82	0.93
Mix 35	92.78	99.78	0.93	92.70	99.78	0.93
Mix 36	93.07	99.81	0.93	93.07	99.83	0.93
Mix 37	93.27	99.89	0.93	93.27	99.90	0.93

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كفاءة نشاط بعض مخاليط مضادات الأكسدة الطبيعية وتداخلاتها المتعاونة

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[٢] المركز الإقليمي للأغذية والأعلاف – مركز البحوث الزراعية – الجيزة – مصر

الملخص العربي

يزداد النشاط الحيوي لفيتامين ج في وجود بعض مضادات الأكسدة الطبيعية مثل حامض الجاليك وحامض التانيك ،والتي قد تتواجد معا أحيانا ويتم استهلاكها بصورة طبيعية،و تتداخل التفاعلات التآزرية فيما بينها بصورة عالية في الغذاء البشري. وقد كان الهدف من هذه الدراسة هو تحديد أي التركيزات والمخاليط من بين مضادات الأكسدة الطبيعية من فيتامين ج وحامض الجاليك وحامض التانيك من مصادر نباتية ،أكثر قدرة على إنتاج تأثيرات تآزرية مضادة للأكسدة . لذا فقد تم تحضير محاليل فردية من مضادات الأكسدة الطبيعية بتركيزات مختلفة ،و تم عمل مخاليط منها ، كما تم قياس كفاءتها المضادة للأكسدة باستخدام إختبار (٢،٢ ثنائي فينيل ١ بيكريل الهيدرازيل) (DPPH•). وقد تم استخدام مستويات مختلفة من كلٍ من مضادات الأكسدة . ثم تم عمل مقارنات لقياس كفاءة نشاط مضادات الأكسدة بين المجموع الجبري لمضادات الأكسدة الفردية وبين التي تم إختبارها في صورة مخاليط ،وذلك لحساب التأثيرات التآزرية بين مضادات الأكسدة الطبيعية .وقد أظهرت نتائج الدراسة أن المخلوط الذي سجل له اعلى نشاط تآزري مضاد للأكسدة هو المخلوط الثنائي الذي أحتوى على فيتامين ج (تركيز ٥٠٠ ميكرومول /لتر) وحامض التانيك (تركيز ٥٠٠ ميكرومول /لتر). وأثبتت النتائج أن خاصية مضاد الأكسدة في هذا المخلوط كانت متزايدة مقارنةً مع المجموع الجبري لتأثيرات مضادات الأكسدة الفردية ،وهذه التداخلات التفاعلية لمضادات الأكسدة فيما بينها يمكن أن تزيد من تأثيراتها المضادة للأكسدة. والنتائج المتحصل عليها يمكن أن تساهم في تنمية مواصفات المنتجات الغذائية لتكون أعلى تأثيراً في مضاداتها للأكسدة .