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OPTIMIZATION OF THE SYNERGISTIC ANTIOXIDANT EFFECT OF SELECTED PHENOLIC COMPOUNDS (GALLIC ACID, ROSMARINIC ACID and CAFFEIC ACID) AND INVESTIGATION OF THEIR ABILITY TO PREVENT FORMATION OF DNA BASE DAMAGE

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ABSTRACT

Considering the areas of use of phenolic compounds, it is important to determine the concentrations at which they show synergistic and antagonistic interactions for their integration into various systems and their correct use. In this study, the synergistic interaction concentration of rosmarinic acid, gallic acid, and caffeic acid was determined by Folin–Ciocalteu and FRAP methods. The central composite design–response surface methodology was used to determine the optimum concentration for synergistic interaction. As a result of the optimization, caffeic acid, rosmarinic acid, and gallic acid showed synergistic interaction at 7.87 µM, 6.75 µM and 9.42 µM concentrations for Folin–Ciocalteu method; 8.03 µM, 9.34 µM and 6.00 µM concentration for FRAP method respectively. The capacity of phenolic compounds to prevent the formation of DNA base damage products was evaluated by GC–MS/MS. As a result, the synergistic concentration of three phenolics reduces the DNA damage products at 37.17% (FOLIN) and 40.17% (FRAP).

Keywords: Antioxidant, DNA oxidation, optimization, phenolic, synergistic effect

SEÇILMIŞ FENOLIK BILEŞIKLERIN (GALLİK ASİT, ROSMARİNİK ASİT ve KAFEİK ASİT) SINERJISTİK ANTIOKSIDAN ETKISININ OPTIMIZASYONU VE DNA BAZ HASARI OLUŞUMUNU ÖNLEME YETENEKLERININ ARAŞTIRILMASI

ÖZ

Fenolik bileşiklerin kullanım alanları düşünüldüğünde, sinerjik ve antagonistik etkileşim gösterdikleri konsantrasyonların belirlenmesi, çeşitli sistemlere entegrasyonları ve doğru kullanımları için

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önemlidir. Bu çalışmada, rosmarinik asit, gallik asit ve kafeik asidin sinerjik etkileşim konsantrasyonu Folin–Ciocalteu ve FRAP yöntemleri ile belirlenmiştir. Sinerjik etkileşim için optimum konsantrasyonu belirlemek üzere merkezi kompozit dizayn–yanıt yüzeyi metodolojisi kullanılmıştır. Optimizasyon sonucunda kafeik asit, rosmarinik asit ve gallik asit Folin–Ciocalteu yöntemi için sırasıyla 7.87 µM, 6.75 µM ve 9.42 µM konsantrasyonlarında; FRAP yöntemi için 8.03 µM, 9,34 µM ve 6.00 µM konsantrasyonlarında sinerjik etkileşim göstermiştir. Fenolik bileşiklerin DNA baz hasarı ürünlerinin oluşumunu önleme kapasitesi GC–MS/MS ile değerlendirilmiştir. Sonuç olarak, üç fenoliğin sinerjik konsantrasyonu DNA hasar ürünlerini %37.17 (FOLIN) ve %40.17 (FRAP) oranında azaltmaktadır.
Anahtar kelimeler: Antioksidan, DNA oksidasyonu, optimizasyon, fenolik, sinerjik etki

INTRODUCTION

Free radicals are self-existing, reactive, unstable, and short-lived molecules containing unpaired electrons (Dreher and Junod, 1996). Free radicals can be formed as a by-product of the aerobic organism or by various external effects such as UV radiation, harmful chemicals, air pollution, stress, smoke, drug toxications and metal ions such as copper, nickel, cadmium, iron, mercury, chromium (Buonocore et al., 2010; Ceylan et al., 2018; Kaur and Kapoor, 2001; Munteanu and Apetrei, 2021). The majority of free radicals in living organisms are formed due to partial reduction of the oxygen molecule and are entitled reactive oxygen species (ROS). ROS generally include hydroxyl (*OH), superoxide (O^{2-}), hydroperoxyl (HOO*), peroxyl (ROO*), and alkoxyl (RO*) radicals (Halliwell, 2006; Jiang and Rusling, 2019; Andrés et al., 2023). The increase in cellular ROS levels leads to oxidative stress, damaging cellular elements such as DNA, protein, and ribosome (Dizdaroglu et al., 2002; Srinivas et al., 2019). The damage to DNA can cause cell division, cancer, ageing, inflammation, and degenerative diseases such as Alzheimer, Parkinson, cardiovascular disease, and atherosclerosis (Aybastıer and Demir, 2021; Seal et al., 2020).

Antioxidants prevent oxidation caused by ROS and can scavenge free radicals. Thanks to these properties, antioxidants play a significant role in avoiding various ailments caused by ROS (Tsao, 2010; Ye et al., 2023). Phenolic compounds are considered potent antioxidants, and they have been shown to inhibit cellular oxidative damage. Chlorogenic acid, resveratrol, and caffeic acid, etc., are examples of these compounds and have been reported to reduce cellular ROS levels by effectively reducing DNA damage. In addition to the radical scavenging properties, they have anti-microbial, anti-fungal, anti-mutagenic, and anti-carcinogenic activity and these effects are the result of antioxidant activity (Ferguson, 2001).

Polyphenols are abundant in fruits, vegetables, and some traditional herbs (Zhang et al., 2018). The combinations of phenolic compounds were hypothesized to have more significant antioxidant activity than expected based on their individual effects (Saucier and Waterhouse, 1999). A synergistic effect is called when two or more compounds are greater than the sum of their individual effects. If there are two or more substances together, when they are less than the sum of the individual effects of these substances, then the antagonistic effect is mentioned. Today, a mixture or combination of various drugs is used for the treatment of many diseases at the same time. For this reason, it is important to investigate the synergistic and antagonistic effects of chemical substances. Phenolic compounds show synergistic, additive, and antagonistic effects when mixed in pairs (Tsao, 2015). The synergistic effects of antioxidant polyphenols are not only a defense mechanism against oxidative stress in biological systems but also facilitate application in food systems (Wang et al., 2011). For example, Mohamed, 2011 reported that a mixture of rosemary and green tea extract had a synergistic effect against oxidation of meats. Irwandi et al., 2000 studied the oxidative behavior of rosemary, sage, and citric acid combinations in linoleic acid and palm olein systems and reported significant synergistic effects between them. Similarly, De Guzman et al., 2009 found that the combination of tetrabutyl hydroquinone and butylated hydroxyanisole had synergistic antioxidant

activity on the stability of soy bean oil. By determining synergistic concentrations, suitable systems have been developed for food preservation.

In this study, three of the polyphenols commonly found in foods, rosmarinic acid (RA), gallic acid (GA) and caffeic acid (CA) were selected and their synergistic antioxidant concentration was investigated and the effect of this concentration on DNA oxidation was examined. For this goal, central composite design-response surface methodology (RSM–CCD) was utilized to evaluate the synergistic antioxidant concentration of rosmarinic acid, gallic acid, and caffeic acid. Then, the effect of phenolic compound concentrations with optimum synergistic antioxidant values on DNA oxidation inhibition ability was expressed.

MATERIALS AND METHODS

Materials

RA, GA, CA, Folin–Ciocalteu reagent, 2,4,6-Tris(2-pyridyl)-s-triazine, and Trolox in HPLC-Grade were purchased from Sigma-Aldrich (St. Louis, MO., USA). The 5,6-dihydrothymine (56DHT), 5-hydroxy-5-methylhydantoin (5H5MH), 5-hydroxy hydantoin (5HH), 5-hydroxycytosine (5HC), 2-hydroxyadenin (2HA) and 2,8-dihydroxyadenine (28DHA) were purchased Toronto Research Chemicals (Canada). The 5,6-dihydrouracil (56DHU), 8-hydroxy-2-deoxyguanine (8OHG), and DNA from the calf thymus were purchased Sigma (USA). The 5-formyluracil (5FU) was purchased IS Chemical Technology in China. The 5-hydroxyuracil (5HU), thymine glycol (TG), 5-(hydroxymethyl) cytosine (5HMC), 8-hydroxyadenine (8HA), and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FG) were purchased the National Institute of Standards and Technology (USA). The 5-(hidroxymethyl) uracil (5HMU) and alloxane (Alx) were purchased Titan Biotech (Delhi, India). The 4,6-diamino-5-nitropyrimidine (46D5NP) was purchased Aldrich Chemical (USA). The 4,6-diamino-5-(formylamino) pyrimidine (46D5FP) was purchased from Santa Cruz Biotechnology (USA).

Methods

Experimental design

Chemometric methods were used to determine the optimum concentration at which GA, RA, and CA solutions showed synergistic effects. In order to optimize the concentrations of synergistic effects shown by antioxidants, an RSM–CCD consisting of 20 experiments with five levels and three parameters was designed. Total phenolic content (TPC) and total antioxidant capacity (TAC) analyses were selected in response to RSM–CCD. Design Expert 7.0.0 (Stat–Ease Inc. USA) was employed for ANOVA analysis using TPC (Folin–Ciocalteu) and TAC (FRAP) measurement results for 20 experiments, considering the levels given in Table 1.

Table 1. Independent factors and their levels used for central composite design

Independent factor	Level				
	-1.732	-1	0	1	1.732
CA (μM)	2.54	4	6	8	9.46
RA (μM)	2.54	4	6	8	9.46
GA (μM)	2.54	4	6	8	9.46

CA, Caffeic acid; RA, Rosmarinic acid; GA, Gallic acid

The number of experiments (N) is estimated using the following Equation 1.

$$N = 2k + 2k + x_0 \quad (1)$$

The coded values of the CCD are given in Table 2, and the predicted responses were calculated using a second-order polynominal Equation 2.

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} x_i x_j \quad (2)$$

Sample preparation for experimental design.

The GA, CA, and RA solutions (100 μM) prepared with methanol and solutions stored at 4°C. The ternary combination to be used in the study was prepared by dilution of 100 μM stock solution according to the concentrations given in Table 1. The solutions were then analysed by

mixing at concentrations corresponding to the coded values given in Table 2.

Table 2. Central composite design of factors with coded values

Treatment	Factors		
	x_1	x_2	x_3
	CA (μM)	RA (μM)	GA (μM)
1	4	4	4
2	8	4	4
3	4	8	4
4	8	8	4
5	4	4	8
6	8	4	8
7	4	8	8
8	8	8	8
9	2.54	4	4
10	9.46	4	4
11	6	2.54	4
12	6	9.46	4
13	6	6	2.54
14	6	6	9.46
15	6	6	6
16	6	6	6
17	6	6	6
18	6	6	6
19	6	6	6
20	6	6	6

CA, Caffeic acid; RA, Rosmarinic acid; GA, Gallic acid

Total phenolic content (TPC) analysis.

The TPC was measured by the Folin–Ciocalteu method. Briefly, x mL of sample or standard, 2-x mL of distilled water, 2.5 mL of Lowry C solution (50:1 ratio of Lowry A: Lowry B, Lowry A; 0.4% NaOH and 0.2% Na_2CO_3 and Lowry B; 0.5 g of $CuSO_4$ and 1 g of $NaKC_4H_4O_6$) and 0.25 mL of Folin–Ciocalteu reagent were mixed (total volume of 4.75 mL) and then incubated 30 min in the dark. The sample's absorbance was measured by a UV–Vis Spectrophotometer (at 750 nm) (Varian Cary 50 Conc). The TPC of GA, RA and CA represent as mg gallic acid equivalent (GAE) (Karkar and Şahin, 2022).

Total antioxidant capacity (TAC) analysis.

FRAP method was used for TAC determination. Trolox solution prepared at increasing concentrations was used as the standard material for the calibration graph. In this method, 10 mM TPTZ solution (40 mM HCl, 20 mM $FeCl_3$ in distilled water) and pH 3.6 acetate buffer were prepared. The x mL of sample or standard and 3-x mL of FRAP reagent (acetate buffer: $FeCl_3$: TPTZ, 10:1:1) were mixed (total volume 3 mL) and then incubated in the dark for 30 min. The sample's absorbance was measured by a UV–Vis spectrophotometer (at 593 nm). The TAC of GA, CA, and RA represent mg Trolox equivalent (TE) (Şahin and Karkar, 2019).

GC-MS/MS analysis of optimum synergistic concentration of GA, CA, and RA.

The ability of the combined synergistic antioxidant activity of the three phenolic compounds to prevent DNA base oxidation was investigated by determining the concentration of DNA base damage products using GC–MS/MS. The DNA base damage products were delicately examined by SRM mode based on product ions and measured quantitatively. The oxidatively damaged DNA products were measured in control (DNA with the Fenton reaction) and test samples (gallic acid, caffeic acid, and rosmarinic acid with the DNA and Fenton reaction). The DNA from the calf thymus (5 mg in 10 mL of ultra-pure water) was incubated at 4℃ for 12 h, and the DNA solution was diluted at a 1:10 ratio and incubated at 4℃ for 12 h. The concentration was determined by UV–Vis spectrophotometer at 260 nm and was calculated using Equation 3.

$$\mu g\ DNA = A_{260} \times 50 \quad (3)$$

The Fenton reaction generated the oxidative stress medium. The Fenton reaction agent formed 300 μM H_2O_2 and 150 μM Fe^{2+} solutions. Three samples (2.5 mL) were arranged to examine the antioxidant activity of CA, GA, and RA (optimum concentration of Folin and FRAP) on DNA oxidation: (1) Fenton and DNA; (2) Fenton, DNA, CA, GA and RA concentrations of Folin; (3) Fenton, DNA, CA, GA and RA concentrations of FRAP. The samples were

incubated for 20 min at 37°C and frozen (-20°C for 18 h) and lyophilized (24 h) in a FreeZone Labconco (MO, USA) (-85°C, at a vacuum of 0.1 mbar). The samples were hydrolyzed with formic acid (1 mL, 60% v/v) (130°C for 30 min) (Şahin and Karkar, 2019).

The DNA base damage products were analyzed by GC–MS/MS (Trace 1300 GC and TSQ 8000 Evo from Thermo Scientific (USA). The column was Agilent Durabond DB-5MS (12 m × 0.20 mm, 0.33 μm), and the flow rate was 1 mL/min. GC–MS/MS was used with electron impact ionization mode. The ion source and MS transfer line temperature were selected as 250°C and 280°C, respectively. Before the analysis, samples were derivatized with BSTFA containing 1% concentration of TMCS (Dawbaa et al., 2017).

Statistical analysis.

All experimental values were analyzed in triplicate and presented as mean value ± standard deviation (SD). According to the ANOVA results, factors with $p<0.05$ are considered significant, and parameters with $p>0.05$ are considered insignificant.

RESULTS AND DISCUSSION

Fitting of models

The synergistic effect concentrations demonstrated by the antioxidants were optimized using an RSM–CCD. The most convenient combination of variables, including GA (2.54–9.46 μM), RA (2.54–9.46 μM), and CA (2.54–9.46 μM) solution was investigated for a synergistic effect. The experimental and predicted responses were given in Table 3.

Table 3. Central composite design of factors with experimental and predicted values

Treatment	Total phenolic content (mg GAE)		Total antioxidant capacity (mg TE)	
	FOLIN		FRAP	
	Experimental	Predicted	Experimental	Predicted
1	22.37	25.28	28.39	30.54
2	25.29	26.36	36.40	36.93
3	20.47	21.46	31.78	31.51
4	32.61	35.74	37.29	36.07
5	23.95	23.85	32.62	33.82
6	29.40	31.44	38.40	38.66
7	29.17	31.13	41.70	41.16
8	51.79	51.91	44.36	44.19
9	20.12	18.28	34.12	32.65
10	39.41	37.21	39.30	40.80
11	23.99	22.05	40.20	37.80
12	38.58	36.48	41.00	43.42
13	32.49	29.29	28.26	27.57
14	42.90	42.06	36.72	37.43
15	27.99	29.69	27.40	27.22
16	29.17	29.69	25.84	27.22
17	28.53	29.69	26.62	27.22
18	34.00	29.69	26.54	27.22
19	29.80	29.69	27.56	27.22
20	28.66	29.69	29.37	27.22

GAE, Gallic acid equivalent; TE, Trolox equivalent

Among the 20 experiments, including five replicates, experiment 8 (CA concentration 8 μM; RA concentration 8 μM; and GA concentration 8 μM) had the highest TPC (51.79 mg GAE), and experiment 9 (CA concentration 2.54 μM; RA concentration 6 μM; and GA concentration 6 μM) the least TPC (20.12 mg GAE) were displayed. The experiment 8 (CA concentration 8 μM; RA concentration 8 μM; and GA concentration 8 μM) had the most significant amount of TAC (44.36 mg TE), and experiment 16 (CA concentration 6 μM; RA concentration 6 μM; and GA concentration 6 μM) had the smallest amount of TAC (25.84 mg TE). The model F-value (15.28 and 20.38 for Folin, and FRAP methods, respectively) was significant at a 95% confidence interval (Table 4).

Table 4. Analysis of variance (ANOVA) for the fitted quadratic polynominal model for optimization of concentration parameters

Source	FOLIN (R^2 = 0.9322)					FRAP (R^2 = 0.9483)				
	DF	SS	MS	*F* value	*p* value	DF	SS	MS	*F* value	*p* value
Model	9	1098.21	122.02	15.28	<0.0001	9	687.11	76.35	20.38	<0.0001
Lack of fit	5	55.72	11.14	2.31	0.1900	5	29.96	5.99	4.00	0.0773
Pure error	5	24.14	4.83			5	7.50	1.50		

DF, Degree of freedom*; SS,* sum of squares; *MS* mean square

RSM analysis of TPC

The RSM analysis in Table 4 displayed a good regression value (R^2=0.9322) and showed the interaction between the TPC and factors of CA concentration, RA concentration, and GA concentration. A significant quadratic polynominal equation for the TPC amount is given in Table 5.

Table 5. Second order polynominal equations and regression coefficients of the response variables (x_1, the concentration of caffeic acid; x_2, the concentration of rosmarinic acid; x_3, the concentration of gallic acid)

Responses	Second order polynominal equations
FOLIN (mg GAE)	$y = 29.69 + 5.47x_1 + 4.16x_2 + 3.69x_3 + 3.30x_1x_2 + 2.77x_2x_3 + 2.00x_3^2$
FRAP (mg TE)	$y = 27.22 + 2.35x_1 + 1.62x_2 + 2.85x_3 + 1.59x_2x_3 + 3.17x_1^2 + 4.46x_2^2 + 1.76x_3^2$

GAE, Gallic acid equivalent; TE, Trolox equivalent

The x_1 (CA concentration), x_2 (RA concentration), x_3 (GA concentration), x_1x_2, x_2x_3, and x_3^2 factors were the most important for the TPC. However, x_1x_3, x_1^2, and x_2^2 had less effect on the TPC. The interaction between factors and TPC was demonstrated by response surface plots in Figures 1a and 1b. The TPC was increased with increasing CA concentration at the high RA concentration. The highest TPC was noticed at higher RA concentrations and higher CA concentrations. Therewithal in Figure 1b, when the GA concentration was scaled up from 2.54 to 9.46 μM, and the RA concentration was kept high, the TPC was observed as high.

RSM analysis of TAC

The RSM analysis of the data in Table 4 indicated that the interaction between the TAC amount and the factors was quadratic with a good regression coefficient (R^2=0.9483). According to ANOVA analysis, the most significant factors (p<0.05) were x_1 (CA concentration), x_2 (RA concentration), x_3 (GA concentration), x_2x_3, x_1^2, x_2^2, x_3^2, and the least effective factors were x_1x_2, x_1x_3 (p>0.05) on the TAC capacity (Table 5). Response surface plots in Figure 1c demonstrated the interaction between factors and TAC. Figure 1c represents the effect of GA concentration, RA concentration, and their mutual interaction on the TAC. The increase in TAC was noticed with

increasing GA concentration. A decrease in the TAC was noticed with increasing RA concentration at first, but when the RA concentration reached 6 μM and antioxidant capacity increased.

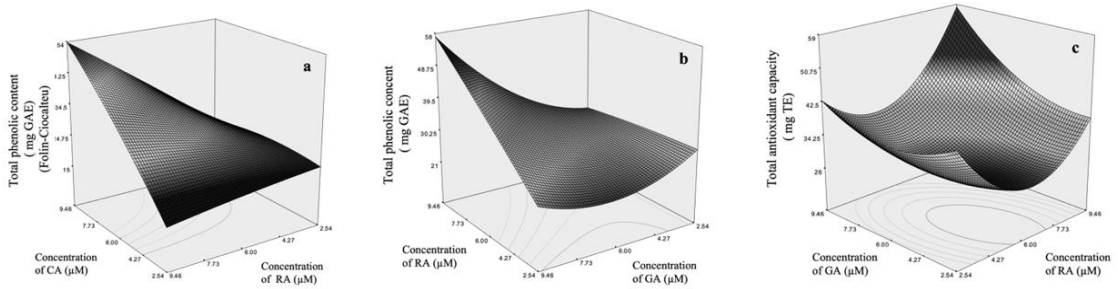

Figure 1. Response Surface Plots of synergistic effect showing the effects of a) concentration of CA and RA on the total phenolic content, b) concentration of RA and GA on the total phenolic content, c) concentration of GA and RA on the total antioxidant capacity (CA: caffeic acid, RA: rosmarinic acid, GA: gallic acid, GAE: Gallic acid equivalent, TE: Trolox equivalent).

Optimization of the synergistic effect of antioxidant

The optimum conditions (predicted and experimental response) for synergistic effect are presented in Table 6. The concentration range of CA 6.14–8.03 μM, RA 2.56–9.34 μM, and GA 6.00–9.42 μM generated the optimum total phenolic content (51.46±4.92 mg GAE) and total antioxidation capacity (45.99±1.41 mg TE). The predicted response agreed with the experimental response obtained using the optimum concentration. This agreement was confirmed by a good correlation coefficient (R^2=0.9322 in Folin and R^2=0.9483 in FRAP). Consequently, the CCD model was considered accurate and confidential for estimating the TPC and TAC for a synergistic effect.

Table 6. Optimum conditions predicted and experimental values of responses

Responses	Maximum values		Optimum concentration of antioxidant		
	Predicted	Experimental	CA (μM)	RA (μM)	GA (μM)
FOLIN (mg GAE)	53.48	51.46±4.92	7.87	6.75	9.42
FRAP (mg TE)	47.27	45.99±1.41	8.03	9.34	6.00

CA, Caffeic acid; RA, Rosmarinic acid; GA, Gallic acid; GAE, Gallic acid equiavalent; TE, Trolox equiavalent

The hydroxyl groups (with number and position) in phenolic compounds and the level of conjugation of the whole molecule are influential in the TAC. The TAC values of CA, GA, and RA are 1.23, 2.63, and 2.65 mg/TE, respectively (Berker et al., 2010). Accordingly, the antioxidant capacity values of GA and RA are close to each other in the same concentration. When the Folin and FRAP results of phenolic compounds (6 μM) were given in Table 7, it was shown that the highest value was obtained for gallic acid. This was consistent with the TAC values for phenolic substances at the same concentration. Looking at the response surface graphs for TPC in Figure 1a, the maximum TPC value was observed when RA concentration was the highest and CA concentration was the lowest. Similarly, in Figure 1b, the maximum TPC value is observed when the concentration of GA is the highest, and the concentration of rosmarinic acid is the lowest. In the environment where GA and RA are present together, the TPC values may decrease due to the steric hindrance that occurs with the increase in the concentration of the compounds.

Table 7. Antioxidants synergistic/antagonistic effect

Phenolic compound	Total phenolic content Folin (mg GAE)	Total antioxidant capacity FRAP (mg TE)
CA (μM)	13.33	6.09
RA (μM)	11.98	6.59
GA (μM)	18.81	22.06
Total	44.12	34.74
Optimum value	51.46	45.99
Interaction	Synergistic	Synergistic

GAE, Gallic acid equivalent; TE, Trolox equivalent; CA, Caffeic acid; RA, Rosmarinic acid; GA, Gallic acid

When Figure 1c is examined, the TAC value is also maximum at the highest concentration of RA and GA. Considering the optimization results (Table 6), the concentrations of phenolic substances for maximum phenolic substance/antioxidant capacity are different because the reaction conditions for both Folin and FRAP methods and the ability of phenolic substances to respond to this reaction are different. This is explained by the chemical structure of phenolic compounds and their steric hindrance each other. According to Table 7, the optimum concentration values were higher than the total results individually. Accordingly, the optimum concentration values results indicate that the antioxidant substances interact synergistically.

Similar to our study, Hajimehdipoor et al., 2014 investigated the synergistic antioxidant effects of four phenolic compounds, caffeic acid, gallic acid, rosmarinic acid and chlorogenic acid and two flavonoids, rutin and quercetin by FRAP method. The synergistic effect was evaluated by comparing the experimental antioxidant activity of the mixtures with the calculated theoretical values and the interactions of the compounds were determined. The results showed that the combination of gallic acid and caffeic acid showed significant synergistic effects (137.8%), while the other combinations were less strong. Gallic acid and rosmarinic acid showed 19.7% synergistic effect, while caffeic acid and rosmarinic acid showed 37.5% synergistic effect. In addition, the triple combination of gallic acid, caffeic acid and rosmarinic acid showed 13.7% synergistic effect. The synergistic effect between gallic acid and caffeic acid decreased with the addition of rosmarinic acid. Skroza et al., 2022 reported that gallic acid has the highest FRAP value among the protocatechuic acid, gentisic acid, gallic acid, vanillic acid, syringic acid and proposed that the highest reducing power of gallic acid may be related to its chemical structure and three -OH groups at positions 3, 4 and 5. They also reported that caffeic and rosmarinic acid had the highest FRAP values among *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid and rosmarinic acid and that this high activity may be related to the structure of rosmarinic acid (two phenolic rings with two -OH groups in the ortho position and an unsaturated double bond and -COOH) and the structure of caffeic acid (catechol structure and distance between -COOH group and functional groups). They also found synergistic interaction between caffeic and rosmarinic acid.

Evaluation of the synergistic effects of antioxidants against DNA oxidative base damage by GC–MS/MS

The chromatograms of the control and test samples are displayed in Figures 2, 3, and 4. The amounts of oxidative DNA base damage products are given in Table 8. Some DNA base damage products were symbolized as below the limit of detection (< LOD) in Table 8, for they were not detected by GC–MS/MS.

A total concentration of 178.93 ng of damaged products/mg DNA was found in the DNA (with the Fenton reaction) sample. The leading cause of oxidative DNA base damage products is hydroxyl free radicals generated by the Fenton reaction. The 7.87 μM CA, 6.75 μM RA, and 9.42 μM GA (Folin–Ciocalteu) were added to the DNA (with the Fenton reaction) sample and a total

concentration of 112.43 ng of damaged products/mg of DNA damage was discovered. According to this result, the DNA damage was reduced by 37.17%. The 8.03 μM CA, 9.34 μM RA, and 6.00 μM GA (FRAP) were added to the DNA (with the Fenton reaction) sample and a total concentration of 107.06 ng of damaged products/mg of DNA damage was obtained. Consequently, DNA damage was reduced by 40.17%. Phenolic compounds have a preventive effect on DNA oxidation due to their high antioxidant activity (Kaur et al., 2019; Nile and Park, 2014; Salar and Purewal, 2017). For example, the inhibitory effect of extracts containing gallic acid and caffeic acid on DNA oxidation has been frequently reported by authors (Chandrasekara and Shahidi, 2012; Kaur et al., 2018; Sudha et al., 2016). Aklan and Aybastıer, 2024 investigated the effect of the extract obtained from *Cichorium intybus* L. on the formation of oxidative DNA base damage products by GC–MS/MS. Accordingly, they found that the formation of DNA base damage products decreased by more than 75% when they used 25 μL of *Cichorium intybus* L. extract, more than 76% when they used 50 μL of extract, and more than 82% when they used 100 μL of extract. *Cichorium intybus* L. extract is rich in phenolic compounds (epigallocatechin gallate, procyanidin B2 and A2, neohesperidin, rosmarinic acid, caffeic acid phenethyl ester, rutin and myricetin). The effect of phenolic compounds with high antioxidant activity on preventing DNA oxidation has been clearly demonstrated. In the results of our study, it was determined that gallic acid, caffeic acid and rosmarinic acid had an inhibitory effect on DNA oxidation in accordance with the literature.

Table 8. The amounts of oxidative DNA base damage products

DNA base damage products	Retention time (min)	DNA	Opt.FOLIN	Opt.FRAP
56DHT	4.79	<LOD	0.24±0.07	<LOD
56DHU	4.81	<LOD	<LOD	<LOD
5H5MH	4.94	134.54±27.60	19.84±0.29	57.33±1.32
5HH	5.17	32.25±4.64	24.01±0.04	14.10±0.89
5FU	5.95	<LOD	2.47±0.07	2.59±0.25
5HU	6.22	0.85±0.06	1.15±0.03	0.54±0.09
5HMU	7.33	0.38±0.54	44.37±0.67	16.10±0.27
Alx	7.39	4.00±0.37	11.35±0.14	3.51±0.01
5HC	7.57	2.32±0.01	2.35±0.01	7.60±0.02
46D5NP	7.74	1.07±0.01	1.30±0.04	1.27±0.01
TG	8.2	0.54±0.08	1.87±0.09	1.04±0.07
5HMC	8.34	<LOD	0.50±0.02	<LOD
46D5FP	9.76	<LOD	<LOD	<LOD
8HA	11.29	<LOD	<LOD	<LOD
2HA	12.47	<LOD	<LOD	<LOD
FG	12.7	2.97±0.01	2.97±0.01	2.97±0.01
28DHA	13.5	<LOD	<LOD	<LOD
8OHG	14.01	<LOD	<LOD	<LOD
Total	-	178.93	112.43	107.06

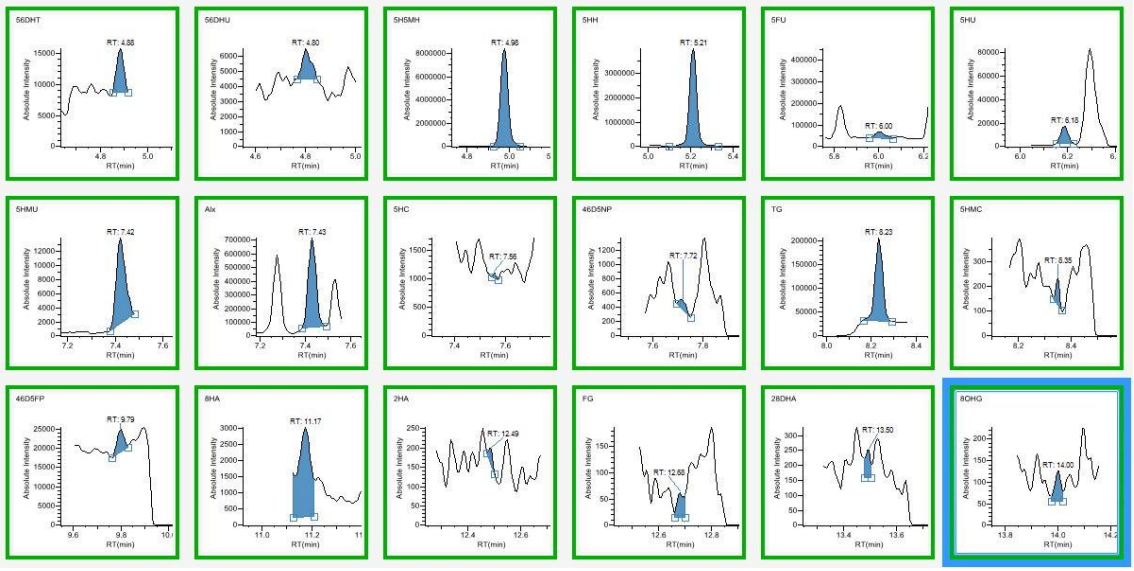

Figure 2. DNA damage product with Fenton oxidation. 56DHT: 5,6-dihydrothymine, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5HC: 5-hydroxycytosine, 2HA: 2-hydroxyadenin, 28DHA: 2,8-dihydroxyadenine, 56DHU: 5,6-dihydrouracil, 8OHG: 8-hydroxy-2-deoxyguanine, 5FU: 5-formyluracil, 5HU: 5-hydroxyuracil, TG: thymine glycol, 5HMC: 5-(hydroxymethyl) cytosine, 8HA: 8-hydroxyadenine, and FG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 5HMU: 5-(hidroxymethyl) uracil, Alx: alloxane, 46D5NP: 4,6-diamino-5-nitropyrimidine, 46D5FP: 4,6-diamino-5-(formylamino) pyrimidine.

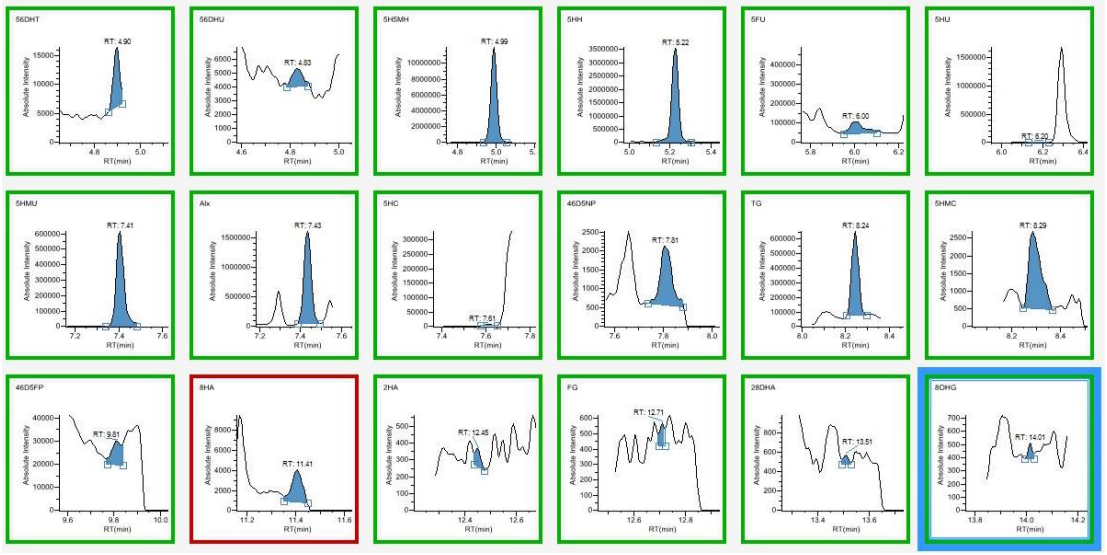

Figure 3. Chromatograms of DNA base damage products (at optimum concentrations determined by Folin analysis). 56DHT: 5,6-dihydrothymine, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5HC: 5-hydroxycytosine, 2HA: 2-hydroxyadenin, 28DHA: 2,8-dihydroxyadenine, 56DHU: 5,6-dihydrouracil, 8OHG: 8-hydroxy-2-deoxyguanine, 5FU: 5-formyluracil, 5HU: 5-hydroxyuracil, TG: thymine glycol, 5HMC: 5-(hydroxymethyl) cytosine, 8HA: 8-hydroxyadenine, and FG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 5HMU: 5-(hidroxymethyl) uracil, Alx: alloxane, 46D5NP: 4,6-diamino-5-nitropyrimidine, 46D5FP: 4,6-diamino-5-(formylamino) pyrimidine.

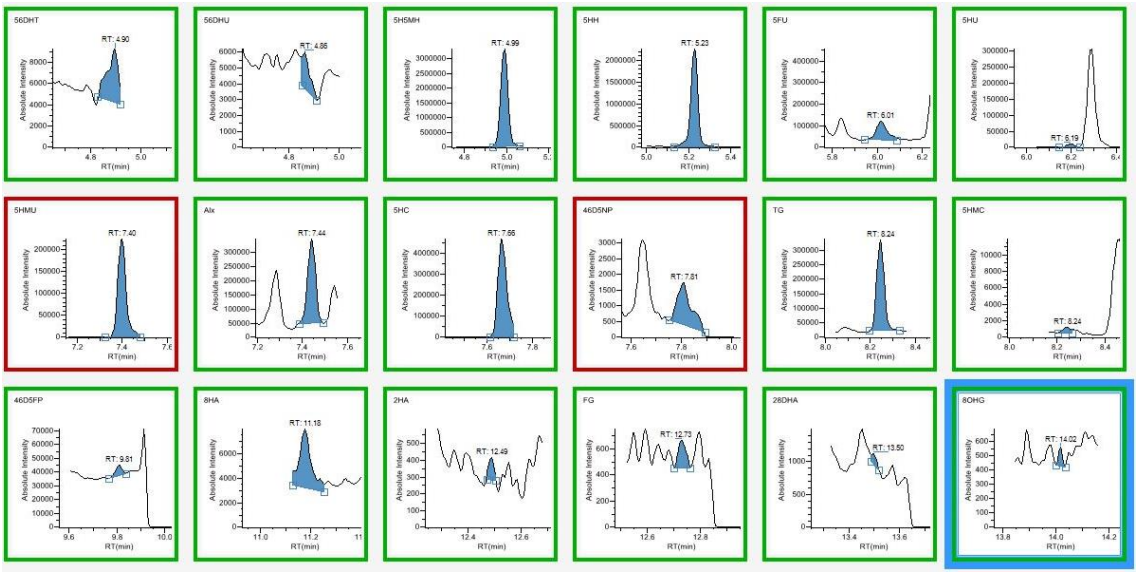

Figure 4. Chromatograms of DNA base damage products (at optimum concentrations determined by FRAP analysis). 56DHT: 5,6-dihydrothymine, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5HC: 5-hydroxycytosine, 2HA: 2-hydroxyadenin, 28DHA: 2,8-dihydroxyadenine, 56DHU: 5,6-dihydrouracil, 8OHG: 8-hydroxy-2-deoxyguanine, 5FU: 5-formyluracil, 5HU: 5-hydroxyuracil, TG: thymine glycol, 5HMC: 5-(hydroxymethyl) cytosine, 8HA: 8-hydroxyadenine, and FG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 5HMU: 5-(hidroxymethyl) uracil, Alx: alloxane, 46D5NP: 4,6-diamino-5-nitropyrimidine, 46D5FP: 4,6-diamino-5-(formylamino) pyrimidine.

CONCLUSION

Phenolic compounds have many different applications in various sectors such as food, medicine and cosmetics due to their high antioxidant activity. Various applications of phenolic compounds are not limited to a single phenolic compound, but combined applications are also very popular. The interaction of phenolic compounds in combined applications is highly effective on the desired antioxidant activity. Therefore, it is very important to determine at which concentrations phenolic compounds show synergistic or antagonistic antioxidant activity. This study revealed the synergistic antioxidant activities of rosmarinic acid, caffeic acid and gallic acid phenolics. A mixture of rosmarinic acid, caffeic acid and gallic acid was found to have a reducing effect on the formation of oxidative base damage products caused by the hydroxyl radical formed as a result of the Fenton reaction in calf thymus DNA at the concentration where the total antioxidant properties were maximum. The results obtained provide information that maximum antioxidant activity will be obtained at the concentrations determined in the study during the application of these three phenolic compounds to various systems.

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DECLARATION OF COMPETING INTEREST

The authors declare to have no competing interests in this article.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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