POTENTIAL OF UV-VIS SPECTROSCOPY FOR DETERMINING THE MECHANISM OF THE SYNERGISTIC ANTIOXIDANT PROCESS OF KAEMPFEROL WITH THREE OTHER FLAVONOIDS AND β-CAROTENE[**]

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The antioxidant activities of flavonoid mixtures can be used to investigate the synergistic antioxidant mechanism of flavonoids. The antioxidant capacities of three flavonoids (quercetin, baicalein, and daidzein) and β-carotene in binary and ternary mixtures with kaempferol were analyzed using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition assay by means of absorption spectroscopy. The results showed that the number of hydroxyl groups and o-hydroxyl groups had a significant effect on the antioxidant activity of the flavonoids, and the mixture of kaempferol, quercetin, and baicalein showed optimal synergistic antioxidant activity. Compared with quercetin and baicalein, kaempferol had the fastest inhibition rate, and multiple prolonged kinetic processes associated with the scavenging of DPPH radicals occurred in mixtures of kaempferol with the other flavonoids and β-carotene. Kaempferol has a potential synergistic antioxidant effect when mixed with daidzein and β-carotene, and the results suggested that this may be due to the regeneration of kaempferol after antioxidation. By means of classic UV-Vis spectroscopy, reaction details of the synergistic antioxidant process of DPPH radical scavenging by flavonoids can be obtained.

Keywords: *UV-Vis spectroscopy, 2,2-diphenyl-1-picrylhydrazyl, flavonoids, β-carotene.*

ОПРЕДЕЛЕНИЕ МЕХАНИЗМА СИНЕРГЕТИЧЕСКОГО АНТИОКСИДАНТНОГО ВЗАИМОДЕЙСТВИЯ КЕМПФЕРОЛА С ТРЕМЯ ДРУГИМИ ФЛАВОНОИДАМИ И β-КАРОТИНОМ С ИСПОЛЬЗОВАНИЕМ УФ-ВИДИМОЙ СПЕКТРОСКОПИИ

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Антиоксидантная активность смесей флавоноидов использована для исследования механизма их синергетического антиоксидантного взаимодействия. Антиоксидантные свойства трех флавоноидов (кверцетина, байкалеина и даидзеина) и β-каротина в бинарных и тройных смесях с кемпферолом охарактеризованы с использованием анализа ингибирования 2,2-дифенил-1-пикрилгидразила (DPPH) с применением абсорбционной спектроскопии. Показано, что количество гидроксильных и о-гидроксильных групп оказывает значительное влияние на антиоксидантную активность флавоноидов, а смесь кемпферола, кверцетина и байкалеина демонстрирует оптимальную синергетическую антиоксидантную активность. По сравнению с кверцетином и байкалеином у кемпферола самая высокая скорость ингибирования, и в смесях кемпферола с другими флавоноидами и β-каротином происходят множественные кинетические процессы, связанные с удалением радикалов DPPH. Кемпферол обладает потенциальным синергетическим антиоксидантным свойством при смешивании с даидзеином и β-каротином, что может быть связано с регенерацией кемпферола после анти-

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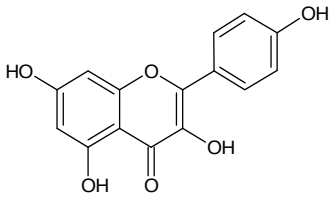
оксидантного действия. С помощью классической УФ-видимой спектроскопии можно получить детали реакции синергетического антиоксидантного процесса удаления радикалов DPPH флавоноидами.

Ключевые слова: *УФ-видимая спектроскопия, 2,2-дифенил-1-пикрилгидразил, флавоноиды, β-каротин.*

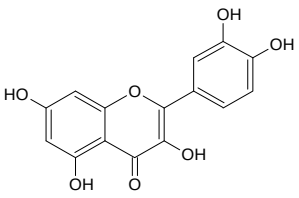
Introduction. Flavonoids, also known as bioflavonoids, are members of a class of low-molecular-weight natural plant components that are primarily found in some colored plants. They cannot be synthesized by the human body and need to be ingested. Clinical studies have shown that flavonoid supplementation via food-derived plants, such as vegetables and fruits, can reduce the risk of cancer and cardiovascular and neurological diseases [1]. The health-promoting effects of flavonoid compounds are believed to be derived from their antioxidant capacity. Flavonoid antioxidants can use the reducing power of phenolic hydroxyl groups to scavenge reactive oxygen species (ROS) directly through hydrogen transfer or electron transfer mechanisms and indirectly by regulating enzyme activity or chelating metal ions [2].

In an actual antioxidant system, multiple antioxidants typically act simultaneously. Some researchers have reported synergistic antioxidant effects among flavonoids, such as between kaempferol and myricetin [3], as well as between quercetin and catechin or cyanidin [4]. Flavonoids and other natural extracts, such as flavonoids, α-tocopherol [5, 6], and a mixture of low-concentration carotenoids and polyphenols, resulted in a significant reduction in ROS levels [7]. The synergistic effect between antioxidants is influenced by the proportion of the formulation, type of antioxidants, and reaction systems [8, 9]. Research on the mechanisms of interaction between different types of natural antioxidants can provide a theoretical and experimental basis for the rational combined use of antioxidants.

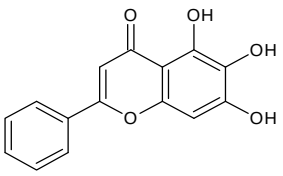
Current experiments on the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals by antioxidants focus on single-wavelength acquisition at ~515 nm, but the absorption peak of flavonoids is generally below 450 nm (e.g., that of kaempferol is at 370 nm). Therefore, this study used steady-state ultraviolet-visible (UV-Vis) absorption spectroscopy to observe the scavenging process of DPPH radicals scavenged by flavonoids to discover detailed changes in waveforms during the scavenging process and to extensively analyze the mechanism of their interactions. We compared the DPPH radical scavenging activities of kaempferol, quercetin, baicalein, daidzein, and β-carotene, which were then added to kaempferol to form a binary or tertiary solution.



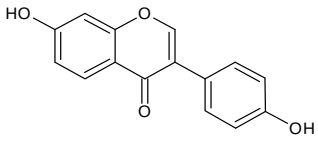
Kaempferol



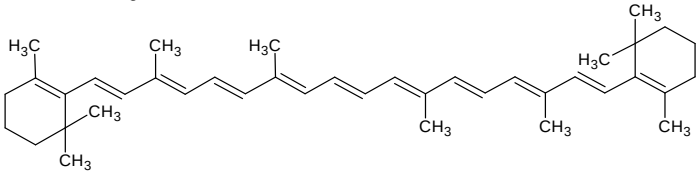
Quercetin



Baicalein



Daidzein



β-carotene

The DPPH radical scavenging activity of the mixtures of flavonoids was tested to determine whether there was a synergistic antioxidant effect between them.

Materials and methods. Kaempferol (≥97%) and quercetin (≥98%) were purchased from Rohn Reagent (Shanghai Eon Chemical Technology Co., Ltd., Shanghai, China). Baicalein (≥98%), daidzein (≥98%), DPPH (≥98%), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$), and β-carotene (≥97%) were purchased from Chongqing Pulico Biotechnology Co., Ltd. (Chongqing, China). Spectroscopically pure ethanol (≥99.8%) was purchased from Aladdin Chemical Reagent (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China).

Kaempferol, quercetin, baicalein, and daidzein were prepared at 0.1 mg/mL (first prepared as 1 mg/mL solution in ethanol and then diluted with ethanol), and β-carotene was prepared at 0.5 mg/mL in ethanol.

Approximately 1 mg of solid DPPH was dissolved in 50 mL of ethanol (stored away from light), then ethanol (0.5 mL) was added to 1 mL of the above solution for dilution. The absorbance was controlled between 0.8–1.0 [10]. The procedure for preparation of the $ABTS^{\bullet+}$ solution and the inhibition calculation method are described in [11].

Equation 1 was used to calculate the percentage of DPPH inhibition:

$$\text{Inhibition DPPH} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right] \times 100\%, \quad (1)$$

where A_{control} (0.8–1.0) is the initial absorbance of the DPPH radical solution and A_{sampel} is the absorbance of the DPPH radical solution at different time intervals after the addition of the sample. The results were also used for IC50 calculations, i.e, the concentration of the sample required to reduce 50% of DPPH radicals.

Preparation of binary and ternary mixed solutions of samples. After obtaining the IC50 values of each flavonoid using Eq. (1), binary and ternary solutions were prepared using half (1/2) or one-third (1/3) of the IC50 values of each flavonoid. Generally, the IC50 values of flavonoids for the scavenging of DPPH and $ABTS^{\bullet+}$ radicals are approximate, but daidzein did not scavenge DPPH radicals at the IC50 value of $ABTS^{\bullet+}$ (Fig. 1S). To avoid adding too much daidzein that would affect the experimental results, the IC50 value of daidzein on $ABTS^{\bullet+}$ radicals was used as the reference concentration for the subsequent preparation of the mixed solution. A volume of 10 μL of 0.5 mg/mL β-carotene solution (~6.21 μM, with no obvious scavenging effect on DPPH radicals) was mixed with kaempferol (at the concentration of its IC50 value) as a binary solution.

Determination of IC50 values and time-continuous steady-state absorption spectra. A volumetric gradient of flavonoid solution (at least 5 volumes, adjusted to 0.5 mL by adding ethanol) was added to the prepared 1.0-mL DPPH radical solution. The total volume of the solution for all experiments was 1.5 mL. After this mixed solution was left to react for 30 min, absorbance was measured at 516 nm (721N, Shanghai Prism Technology Co., Ltd.). The DPPH radical inhibition of the flavonoid solutions at each volume gradient was obtained using Eq. (1). A linear fit of the above data was performed to calculate the amount (concentration) of flavonoids used for 50% DPPH radical inhibition (IC50 value of flavonoids). Three parallel measurements were conducted for each dose. The details of this method are available in the study by Li [10].

The steady-state absorption spectra (Lambda 950, PerkinElmer Instruments Ltd.) were measured immediately after the addition of the prepared kaempferol solution, binary, and ternary mixtures to the DPPH radical solution, which was the absorption spectra of 1 min, followed by the acquisition of absorption spectral data every 3 min up to 43 min.

All measurements were performed at room temperature in a 1 cm cuvette with sealed lids. The lifetime fitting programs for kinetics analyses were calculated using SciDAVis 1.26 (open source).

Results and discussion. *Antioxidant activity of individual flavonoids.* This work included kaempferol, quercetin, baicalein and daidzein. Based on the IC50 values obtained (Fig. 2S, Table 1S), the ability of flavonoids to scavenge DPPH radicals is related to the number of hydroxyl groups; however, the IC50 of baicalein was lower than that of kaempferol, indicating that the hydroxyl groups on the *A*-ring were more capable of deprotonation than those on the *B*-ring. It was also found that quercetin was approximately 2× more powerful than kaempferol in scavenging DPPH free radicals, whereas the two molecules differed only by the hydroxyl group at the 3′ position of the *B* ring, indicating that the hydroxyl group at this position has a significant influence on the antioxidant capacity of flavonoids.

Scavenging of DPPH radicals by kaempferol. The absorbance of the DPPH radical at 516 nm decreased significantly at the beginning of the experiment (within 1 min; Fig. 1a). An elevated absorption peak was observed at 295 nm, which was attributed to the kaempferol-scavenging DPPH radical. A similar absorption peak in the methanol solution was identified by mass spectrometry, representing the $[M–H]^-$ ion of the compound formed in the reaction through the transfer of two H atoms to the DPPH radical, after which the oxidized kaempferol bound two CH_3OH molecules [12]. In our work, ethanol was used, which may have yielded a similar product. The observed rapid rate of DPPH radical scavenging (Fig. 1b) by kaempferol may be due to the positions of its hydroxyl groups – they are not located near each other and do not form intramolecular hydrogen bonds after H transfer to DPPH (as observed in flavonoids with hydroxyl groups at the 3′,4′ positions); therefore, the loss of protons has little effect on the protons of other hydroxyl groups [13]. Details are provided in the Discussion section.

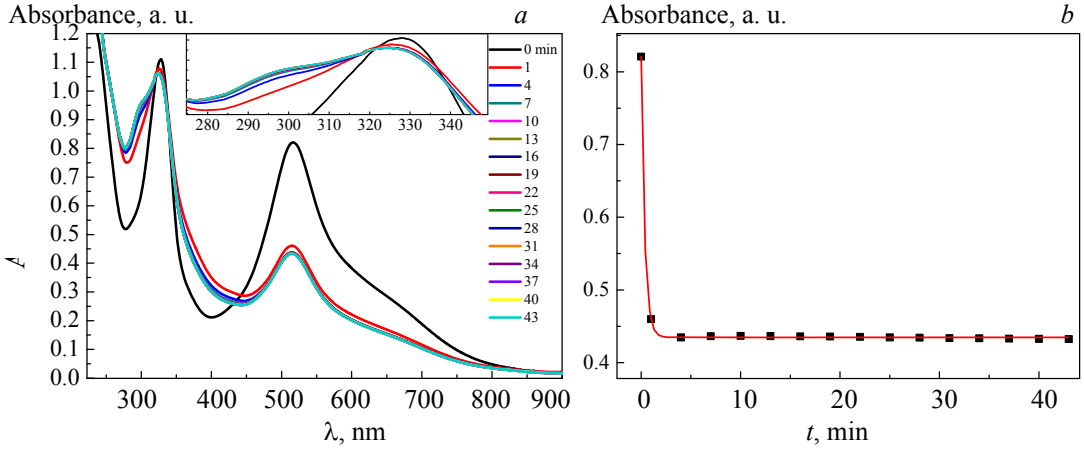


Fig. 1. (a) Steady-state absorption spectra of DPPH radicals scavenged by kaempferol with time (16.11 μM, 0 min represents the steady-state absorption spectrum of DPPH radicals before kaempferol was added); (b) the corresponding kinetic and fitted curves at 516 nm.

Scavenging of DPPH radicals by kaempferol mixed with quercetin, baicalein, and daidzein. A mixture of flavonoids may have synergistic or antagonistic effects [3, 14–16]; therefore, we mixed kaempferol with three other flavonoids with different numbers and positions of hydroxyl groups to investigate whether there might be synergistic or antagonistic antioxidant effects between them.

Scavenging process of DPPH radicals with binary mixtures of quercetin, baicalein, and daidzein. Compared with the results shown in Fig. 1a, the interaction of kaempferol with quercetin resulted in an insignificant peak at 295 nm (Fig. 2a). The absorbance at 516 nm decreased gradually over time, and the DPPH radical scavenging process was prolonged (compared to Fig. 1b). The peak at 295 nm was inconspicuous in the spectrum of the mixture of kaempferol and baicalein (Fig. 2b). A distinct shoulder peak appeared at 370 nm, which was attributed to deprotonation of baicalein [17]. The absorbance at 516 nm decreased significantly within 1 min and slowly decreased with time. The DPPH radical scavenging activity of the mixture of kaempferol and daidzein (Fig. 2c) was significantly lower than that of kaempferol alone. The peak at 295 nm also became inconspicuous, and there was no noticeable increasing trend with a time delay; however, compared with that in Fig. 1a, this absorbance peak became considerably broader and more intense. The increase was attributed mainly to daidzein because it absorbs in this wavelength range. Within 1 min, the absorbance at 516 nm decreased significantly and then decreased slowly with time.

Scavenging process of DPPH radicals by kaempferol with ternary mixtures of quercetin, baicalein, and daidzein. As most studies have focused on binary mixtures of flavonoids, the possible synergistic effects of ternary mixtures of these flavonoids were investigated in this study. The mixture of kaempferol, quercetin, and baicalein had a pronounced synergistic antioxidant effect, and the absorbance at 516 nm was reduced within 1 min (Figs. 3a,d). The absorbance value continued to decline with time, and there was an apparent increase in peak intensity at 290–310 nm, which is the characteristic peak position of the antioxidant product kaempferol; there was also an obscure peak at 370 nm. As shown in Fig. 2b, this peak was mainly attributed to the product of baicalein after deprotonation.

The inhibition of DPPH radicals was enhanced by the addition of either quercetin or baicalein to the daidzein and kaempferol solutions (Figs. 3b,c *vs.* Fig. 2c). The mixture of daidzein and quercetin or baicalein with kaempferol gave rise to an absorption peak of daidzein at 310 nm; moreover, the product peak after kaempferol antioxidation was not apparent, similar to the case of kaempferol mixed with daidzein solution alone (Fig. 2c). The inhibition of DPPH radicals and the kinetics of the peak at 516 nm were similar for both mixed solutions. Comparing Figs. 2b and 3c shows that the solution with the addition of baicalein also gave rise to an absorption peak at 370 nm (the product after deprotonation of baicalein). Notably, the absorption peak of the product of DPPH radical scavenging by kaempferol (290–310 nm) increased in intensity with a time delay; however, this peak elevation was not observed with the addition of quercetin (Fig. 3a) or the binary mixture (Figs. 2a–c), indicating that the interaction of kaempferol with quercetin or baicalein may have changed after the addition of daidzein.

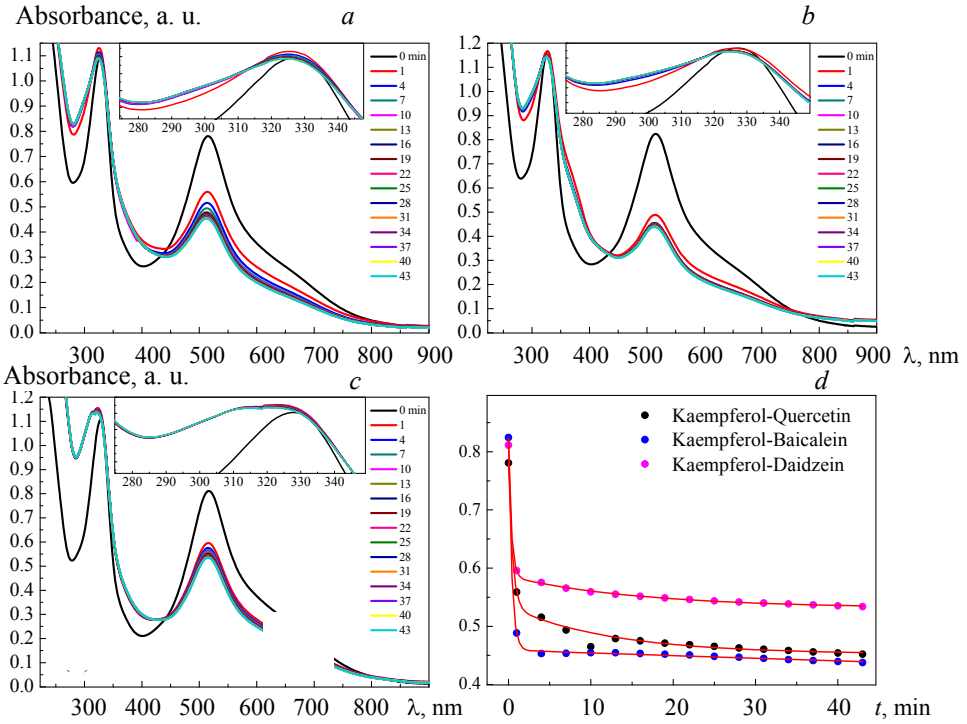


Fig. 2. Steady-state absorption spectra of DPPH radical scavenging by the addition of (a) quercetin, (b) baicalein, and (c) daidzein to kaempferol. (d) The corresponding kinetic and fitted curves at 516 nm. As observed, 0 min represents the steady-state absorption spectrum of DPPH radicals before the kaempferol mixture was added. The binary mixture was mixed at half the IC50 value of each flavonoid.

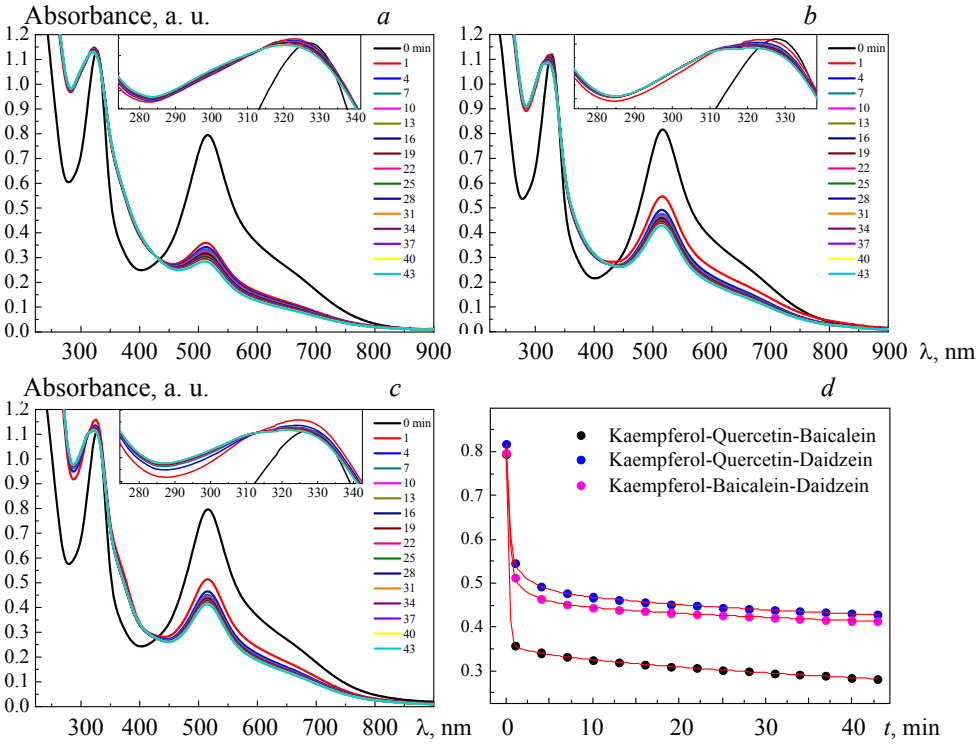


Fig. 3. Steady-state absorption spectra of DPPH radical scavenging by the addition of (a) quercetin and baicalein, (b) quercetin and daidzein, (c) and baicalein and daidzein to kaempferol. (d) The corresponding kinetic and fitted curves at 516 nm. As observed, 0 min represents the steady-state absorption spectrum of DPPH radicals before the kaempferol mixture was added. The ternary mixture was mixed according to one-third of the IC50 value of each flavonoid.

DPPH radical scavenging process in a mixture of kaempferol and β-carotene. The mixture of kaempferol with β-carotene (~6.21 μM) resulted in ~50% inhibition within 1 min (Fig. 4b), and the generation of kaempferol antioxidant products at 295 nm. A gradual increase in the absorption peak of β-carotene was observed (Fig. 4a), likely due to the gradual downward deposition of β-carotene, as it is slightly soluble in ethanol. A similar phenomenon was observed in the experiments with carotene mixed with DPPH radicals (Fig. 3S). The kinetics monitored by the peak at 516 nm approximates that of DPPH radical scavenging by kaempferol (Fig. 1b), except that a longer scavenging process occurred.

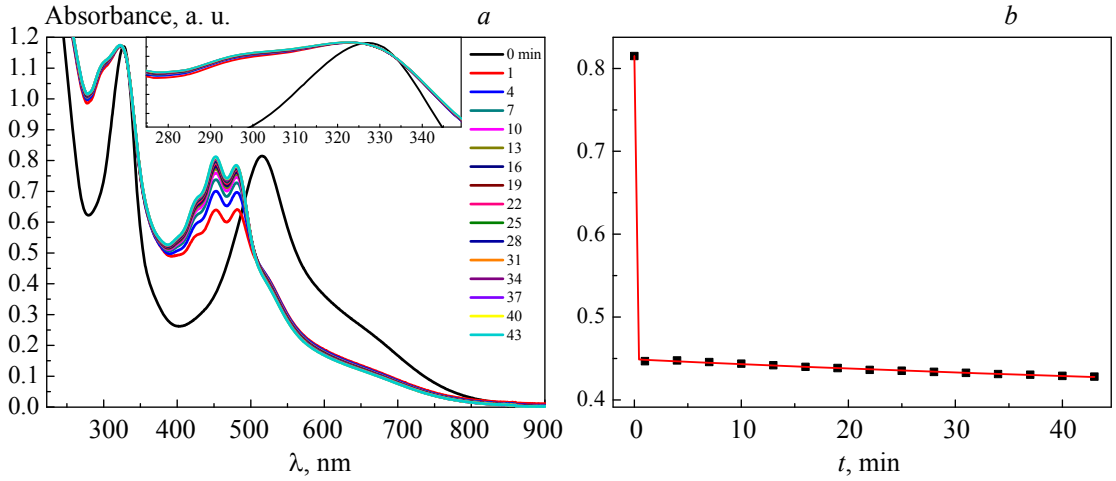


Fig. 4. (a) Steady-state absorption spectra of DPPH radical scavenging by the addition of β-carotene (6.21 μM) to kaempferol. (b) The corresponding kinetic and fitted curves at 516 nm. As observed, 0 min represents the steady-state absorption spectrum of DPPH radicals before the kaempferol mixture was added.

Effect of the number and position of hydroxyl groups of flavonoids on DPPH radical inhibition. It is generally accepted that the greater the number of hydroxyl groups in a flavonoid, the stronger its antioxidant capacity [5, 18] due to the fact that the number of protons that can be transferred increases. As seen from our results, the number of hydroxyl groups in daidzein, baicalein, kaempferol, and quercetin increased; however, the antioxidant capacity of baicalein was greater than that of kaempferol, indicating that the number of hydroxyl groups is not necessarily proportional to the antioxidant capacity. The positions of the hydroxyl groups and the corresponding bond dissociation energies are important factors [13, 19–22]. The *o*-trihydroxyl group of baicalein exhibited a significantly stronger ability to scavenge DPPH radicals than that of kaempferol [23], suggesting that the *o*-trihydroxy group is more susceptible to deprotonation, possibly because the conjugation of the *o*-trihydroxy oxygen atom makes baicalin electronically repulsive, which in turn makes the hydroxyl group more vulnerable to attack [24]. It was observed that the IC50 of quercetin was approximately one-third that of kaempferol, whereas the difference between them was only the 3′-OH group. It is presumed that this 3′-OH group can form an *o*-dihydroxy group with 4′-OH in quercetin, similar to the *o*-trihydroxy group of baicalein, greatly enhancing the antioxidant ability [25]. At a similar concentration, daidzein did not show the ability to scavenge DPPH radicals [26], likely because of its low number of hydroxyl groups and lack of *o*-hydroxy groups. Therefore, it was speculated that an increase in the number of hydroxyl groups and the structure of *o*-hydroxyl groups can enhance the DPPH radical inhibition of flavonoids.

Synergistic scavenging effect of DPPH radicals among flavonoids. A mixture of different flavonoid compounds may have synergistic antioxidant [8, 14, 15], antagonistic [3, 27, 28], or additive effects [4, 29]. The study of mixed flavonoids can provide an in-depth understanding of the mechanism of interaction of different flavonoids and provide a reference for adding flavonoid compounds to mixtures for antioxidant purposes.

It was found that similar concentrations of daidzein did not have a significant scavenging effect on DPPH; however, when mixed with kaempferol (half the IC50 value), it appeared to have little synergistic antioxidant effect (scavenging rate >25%). After adding either baicalein or quercetin to the kaempferol and daidzein mixture, the scavenging rate was close to 50%. These results suggest that daidzein exhibits a syner-

gistic antioxidant capacity with kaempferol or other flavonoids. This may be due to the regenerative effect of daidzein on kaempferol [27, 30] or the formation of stronger antioxidants with other flavonoids [31].

In our study, the mixtures of kaempferol with baicalein and quercetin showed similar DPPH radical inhibition, suggesting that the mixtures of kaempferol with these two flavonoid compounds induced an additive effect (no significant synergistic antioxidant capacity was observed in the mixture of quercetin and baicalein (data not shown)). Inhibition was significantly increased in the kaempferol, quercetin, and baicalein mixture, indicating that this ternary flavonoid mixture had a synergistic antioxidant effect.

The absorption spectra of DPPH radical scavenging by the ternary mixture and the binary mixture were compared, and some spectral differences were observed; for example, the absorption of kaempferol at 295 nm increased with time (Figs. 1a and 3c). The shoulder peaks of the antioxidant products of baicalein at approximately 370 nm are evident in Figs. 2b and 3c, suggesting that there may be subtle differences in the specific mechanisms of antioxidation in the binary and ternary systems of these four flavonoids. However, more detailed experiments are required to elucidate the mechanisms of these interactions.

Effect of flavonoid mixing on the scavenging rate of DPPH radicals. The kinetic lifetime of the peak at 516 nm was fitted (Table 1), and it was found that the aforementioned binary and ternary mixtures all underwent a rapid kinetic decay process (0.22–0.49 min), which occurred in kaempferol alone. Therefore, the process was attributed to the scavenging process of DPPH radicals by kaempferol, and the reason for such a rapid scavenging rate was that there was no *o*-hydroxyl group in kaempferol that could form intramolecular hydrogen bonds to prevent the DPPH radical from approaching its 4′-OH, and the unpaired electrons became highly delocalized and produced ten resonance structures that also increased its rate of scavenging DPPH radicals [13]. In contrast, flavonoids with *o*-dihydroxy and *o*-trihydroxy groups can form intramolecular hydrogen bonds that prevent DPPH radicals from reacquiring protons [13]; therefore, their scavenging rate is relatively low [32] (clearer in baicalein; data not shown). A second long-lived process was observed when kaempferol was mixed with quercetin or baicalein. This lifetime was approximately 12 min with quercetin and was increased with baicalein (limited by the actual experimental time; this lifetime is for reference).

TABLE 1. DPPH Inhibition by Kaempferol (K) and Its Mixture with Quercetin (Q), Baicalein (B), Daidzein (D), and β-carotene (β-Car) and the Fitted Lifetimes of the Peak at 516-nm Observed During the Scavenging of DPPH Radicals

Flavonoid	Mixture*	Inhibition, %	τ_1, min	τ_2, min	τ_3, min
K	1	47.33	0.37 ± 0.0082	–	–
K: Q	1/2: 1/2	42.11	0.49 ± 0.035	12.61 ± 1.67	–
K: B	1/2: 1/2	46.91	0.40 ± 0.011	>1000	–
K: D	1/2: 1/2	34.20	0.36 ± 0.014	16.63 ± 1.28	–
K: Q: B	1/3: 1/3: 1/3	64.59	0.22 ± 0.0089	4.90 ± 0.80	72.66 ± 11.53
K: Q: D	1/3: 1/3: 1/3	47.64	0.28 ± 0.043	2.08 ± 0.13	37.32 ± 1.37
K: B: D	1/3: 1/3: 1/3	48.23	0.34 ± 0.031	2.56 ± 0.28	43.10 ± 4.92
K: β-Car	1: (6.21 μM)	47.45	0.036 ± 0.00	92.65 ± 44.51	–

* The IC50 values of each flavonoid.

Although daidzein did not have significant DPPH scavenging ability during the experimental period of this study, there may be some synergistic antioxidant effect with kaempferol (as discussed in the previous section); thus, a long-life process of approximately 16 min was also observed in the kinetic study. As mentioned in section 4.2, this lifetime may be due to the regenerative effect of daidzein on kaempferol, which has lost protons [30, 33], or the formation process of a stronger antioxidant [31].

For the ternary mixture solution, the kinetic results were consistent with three scavenging processes, except for the first DPPH scavenging by kaempferol, an additional scavenging process of approximately 2–5 min, which may be the result of the interaction of the three flavonoids during the scavenging of DPPH radicals. The ternary mixtures all accelerated the scavenging of kaempferol (compared to binary mixtures), but in practical applications, a higher scavenging rate may not be better; however, for the long-term preservation of food, it is necessary to extend the scavenging time of antioxidants [34].

Synergistic scavenging of DPPH radicals by a kaempferol and β-carotene mixture. Studies have shown that mixtures of carotenoids and various natural plant extracts (flavonoids, resveratrol, and astaxanthin) exhibit synergistic antioxidant activity [35, 36]. There are three possible mechanisms for this phenomenon: β-carotenes regenerate flavonoids. Astaxanthin radical cations react with β-carotene to regenerate astaxanthin, and then astaxanthin continues to scavenge peroxyl radicals [37]; flavonoids regenerate β-carotene, In liposomes, berberoside anions can reduce β-carotene cation radicals, and β-carotene can continue to exert anti-lipid oxidation effects on membranes [38]; mutual protective effects occur. Vitamin E protects β-carotene from oxidation, whereas the oxidation products of β-carotene and retinol can reduce and regenerate vitamin E [39, 40].

In this work, 6.21 μM (10 μL, 0.5 mg/mL) β-carotene had no scavenging effect on DPPH radicals (Fig. 3S) [41] (~270.08 μM β-carotene could scavenge ~50% of DPPH radicals; data not shown). By comparing the absorption spectra obtained during the DPPH scavenging process of kaempferol and the β-carotene mixture with the kaempferol solution alone (Fig. 1a *vs.* Fig. 4a), it was observed that the peak of kaempferol at 280–310 nm, which increased during the scavenging process, remained the same. Therefore, we presume that in both solutions, kaempferol scavenges the DPPH radical. By comparing the kinetics associated with the normalized peak at 295 nm (Fig. 5), we found that when kaempferol interacted with the DPPH radical alone, and it increased to the maximum absorbance within 15 min, indicating that the scavenging process of DPPH radicals was completed within that period. After the addition of β-carotene, the absorbance peak at 295 nm continued to increase slowly, corresponding to the long-lived process related to the peak at 516 nm (Table 1). This indicates that although the addition of carotenoids did not enhance the antioxidant capacity of kaempferol in 1 min, it had a potential synergistic antioxidant capacity with kaempferol over a long period of time because the 295 nm peak is attributed to the antioxidant product of kaempferol. Therefore, it is presumed that this long scavenging process may be because of the regeneration process of kaempferol, which then continues to scavenge DPPH radicals. This kinetic process was not fully captured because of the experimental time of our current study; thus, subsequent longer observations are necessary.

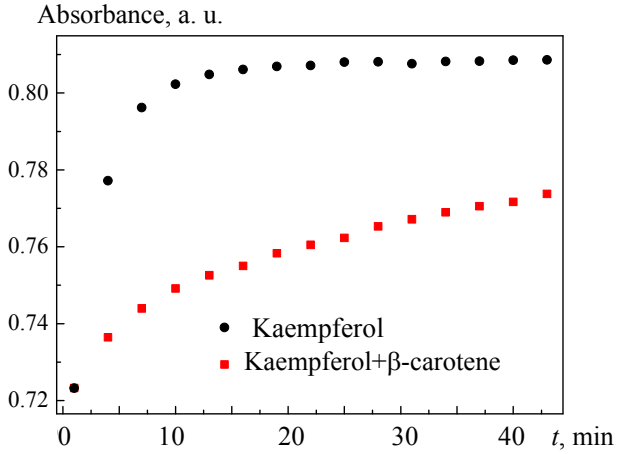


Fig. 5. Kinetics at 295 nm (data after 1 min) during the scavenging of DPPH radicals by kaempferol (•, data from Fig. 1a) and the kaempferol-β-carotene mixture (▪, data from Fig. 4a), normalized at 1 min.

Advantage of UV-Vis spectroscopy in antioxidant activity evaluations. Antioxidant activity evaluations on DPPH radicals are generally measured at ~515 nm (the absorbance of DPPH radicals) [42], but the absorption peaks of flavonoids are mainly below 450 nm, and the single-wavelength information might not include changes in the spectral shape of flavonoids interacting with radicals. More in-depth analysis requires more comprehensive spectral data, and UV-visible spectroscopy can be used to obtain some spectral details. As mentioned in the preceding discussion, the observation of subtle spectral shape changes can be used to explore the mechanism of flavonoid antioxidant action more deeply. Therefore, we conducted multiband UV-visible spectroscopy for individual flavonoids, based on which the mechanism of interaction between flavonoids and β-carotene in the synergistic antioxidant process was investigated as an extended application of classic UV-visible absorption spectroscopy. From these subtle spectral signals, we speculate that the syn-

ergistic antioxidant capacity of kaempferol with other flavonoids/carotenoids may occur mainly as a result of the regeneration of kaempferol. However, in this work, only the scavenging process of DPPH radicals by flavonoids was investigated because DPPH radicals have low absorption values in the absorption peak region of flavonoids and do not cause much interference with the analysis of flavonoid absorption spectral signals; however, the scavenging capacity against DPPH radicals is influenced by the reaction conditions such as solvent and composition of mixed solvents [43, 44], temperature [45, 46], and radical concentration [47]. Therefore, to better understand the antioxidant mechanism, more detailed characterization methods, more types of free radicals and flavonoids, and ultrafast spectroscopy to explore the rapid scavenging process are also necessary.

Conclusions. The application of UV–Vis absorption spectra can provide a more comprehensive view of the free radical scavenging process of flavonoids and a more in-depth understanding of their possible synergistic antioxidant interaction mechanism. The results of the present study showed that the antioxidant capacity of flavonoids was related not only to the number of hydroxyl groups but also to the position of the hydroxyl groups. Flavonoids with *o*-dihydroxy at the 3′,4′ positions and *o*-trihydroxy at the 5, 6, 7 positions had greater antioxidant activity but slowed their DPPH inhibition process – kaempferol had the highest inhibition rate, followed by quercetin and baicalein. Although daidzein and β-carotene did not have a significant scavenging effect on DPPH radicals (at concentrations similar to those of the other flavonoids in this study), they showed potential synergistic antioxidant effects when mixed with kaempferol. This synergistic antioxidant effect may be due to the regenerative effect of kaempferol after antioxidation. A mixture of kaempferol, quercetin, and baicalein exhibited optimal synergistic antioxidant activity; however, the exact underlying mechanism requires further investigation.

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REFERENCES

1. G. Galati, P. J. O'Brien, *Free Rad. Biol. Med.*, **37**, 287–303 (2004).
2. Q. Wang, Y. T. Wang, S. P. Pu, Y. T. Zheng, *Biochem. Biophys. Res. Commun.*, **324**, 605–610 (2004).
3. M. Hidalgo, C. Sanchez-Moreno, S. de Pascual-Teresa, *Food Chem.*, **121**, 691–696 (2010).
4. A. S. Meyer, M. Heinonen, E. N. Frankel, *Food Chem.*, **61**, 71–75 (1998).
5. S. S. Pekkarinen, I. M. Heinonen, A. I. Hopia, *J. Sci. Food Agric.*, **79**, 499–506 (1999).
6. H. Matsufuji, R. Sasa, Y. Honma, H. Miyajima, M. Chino, T. Yamazaki, T. Shimamura, H. Ukeda, T. Matsui, K. Matsumoto, K. Yamagata, *J. Jpn. Soc. Food Sci. Technol. – Nippon Shokuhin Kagaku Kogaku Kaishi*, **56**, 129–136 (2009).
7. M. Fattore, D. Montesano, E. Pagano, R. Teta, F. Borrelli, A. Mangoni, S. Seccia, S. Albrizio, *J. Food Compos. Anal.*, **53**, 61–68 (2016).
8. D. Skroza, I. G. Mekinic, S. Svilovic, V. Simat, V. Katalinic, *J. Food Compos. Anal.*, **38**, 13–18 (2015).
9. J. P. A. Freitas, F. R. M. Franca, M. S. Silva, R. J. Toms, G. F. da Silva, *Korean J. Chem. Eng.*, **36**, 1298–1304 (2019).
10. X. Li, *Chemistry Select*, **3**, 13081–13086 (2018).
11. X. Li, X. Ouyang, R. Cai, D. Chen, *Molecules (Basel, Switzerland)*, **24**, 1–10 (2019).
12. E. Hvattum, Y. Stenstrom, D. Ekeberg, *J. Mass Spectrom.*, **39**, 1570–1581 (2004).
13. D. I. Tsimogiannis, V. Oreopoulou, *Innov. Food Sci. Emerg. Technol.*, **7**, 140–146 (2006).
14. N. Aftab, A. Vieira, *Phytother. Res.*, **24**, 500–502 (2010).
15. S. S. Wang, D. M. Wang, Z. H. Liu, *Ind. Crop. Prod.*, **67**, 227–238 (2015).
16. G. Mercado-Mercado, L. A. de la Rosa, E. Alvarez-Parrilla, *J. Mol. Struct.*, **1199**, 1–8 (2020).
17. R. Liang, C. H. Chen, X. C. Ai, J. P. Zhang, *Chin. J. Mag. Res.*, **27**, 132–140 (2010).
18. C. L. Tian, X. Liu, Y. Chang, R. X. Wang, T. M. Lv, C. C. Cui, M. C. Liu, *S. Afr. J. Bot.*, **137**, 257–264 (2021).
19. Y. J. Hua, X. C. Li, W. H. Zhang, B. Chen, Y. M. Liu, X. J. Zhao, H. Xie, D. F. Chen, *J. Saudi Chem. Soc.*, **25**, 1–10 (2021).
20. Q. Zhang, W. B. Yang, J. C. Liu, H. Liu, Z. Z. Lv, C. L. Zhang, D. L. Chen, Z. G. Jiao, *Oxidative Med. Cell. Longev.*, 1–12 (**2020**).
21. K. L. Khanduja, A. Bhardwaj, *Indian J. Biochem. Biophys.*, **40**, 416–422 (2003).
22. K. P. Suja, A. Jayalekshmy, C. Arumughan, *J. Agric. Food Chem.*, **52**, 912–915 (2004).
23. Z. H. Gao, K. X. Huang, X. L. Yang, H. B. Xu, *Biochim. Biophys. Acta-Gen. Subj.*, **1472**, 643–650 (1999).

24. S. S. Qiu, C. C. Jiang, R. J. Zhou, C. H. Li, *Chin. J. Struct. Chem.*, **39**, 57–65 (2020).
25. A. G. Veiko, E. A. Lapshina, I. B. Zavodnik, *Mol. Cell. Biochem.*, **476**, 4287–4299 (2021).
26. M. K. Johnson, G. Loo, *Mutat. Res.-DNA Repair*, **459**, 211–218 (2000).
27. E. A. Gonzalez, M. A. Nazareno, *LWT-Food Sci. Technol.*, **44**, 558–564 (2011).
28. J. Valerga, M. Reta, M. C. Lanari, *LWT-Food Sci. Technol.*, **45**, 28–35 (2012).
29. M. Colon, C. Nerin, *Eur. Food Res. Technol.*, **242**, 211–220 (2016).
30. D. Tsimogiannis, A. Bimpilas, V. Oreopoulou, *Eur. J. Lipid Sci. Technol.*, **119**, 1–9 (2017).
31. B. J. F. Hudson, *Food Antioxidants*, Elsevier Applied Science, London (1990).
32. H. Y. Zhang, Y. M. Sung, X. L. Wang, *Chem.-Eur. J.*, **9**, 502–508 (2003).
33. M. N. Peyrat-Maillard, M. E. Cuvelier, C. Berset, *J. Am. Oil Chem. Soc.*, **80**, 1007–1012 (2003).
34. L. Bateman, H. Hughes, A. L. Morris, *Discuss. Faraday Soc.*, **14**, 190–199 (1953).
35. H. J. Wang, R. Liang, L. M. Fu, R. M. Han, J. P. Zhang, L. H. Skibsted, *Food Funct.*, **5**, 1573–1578 (2014).
36. T. Zhang, S. Deng, Y. H. Chen, X. P. Du, *Food and Fermentation Industries*, **47**, 8–15 (2021).
37. J. Liang, Y. X. Tian, F. Yang, J. P. Zhang, L. H. Skibsted, *Food Chem.*, **115**, 1437–1442 (2009).
38. R. M. Han, Y. X. Tian, E. M. Becker, M. L. Andersen, J. P. Zhang, L. H. Skibsted, *J. Agric. Food Chem.*, **55**, 2384–2391 (2007).
39. B. Zhou, Z. Chen, Y. Chen, Z. Jia, Y. Jia, L. Zeng, L. Wu, L. Yang, Z. L. Liu, *Appl. Magn. Res.*, **18**, 397–406 (2000).
40. M. T. Schroeder, E. M. Becker, L. H. Skibsted, *J. Agric. Food Chem.*, **54**, 3445–3453 (2006).
41. L. Muller, K. Frohlich, V. Bohm, *Food Chem.*, **129**, 139–148 (2011).
42. M. C. Foti, *J. Agric. Food Chem.*, **63**, 8765–8776 (2015).
43. S. B. Kedare, R. P. Singh, *J. Food Sci. Technol.*, **48**, 412–422 (2011).
44. M. C. Foti, C. Daquino, C. Geraci, *J. Org. Chem.*, **69**, 2309–2314 (2004).
45. V. Thavasi, R. P. A. Bettens, L. P. Leong, *J. Phys. Chem. A*, **113**, 3068–3077 (2009).
46. K. Sak, *Mini-Rev. Med. Chem.*, **14**, 494–504 (2014).
47. L. M. Magalhães, M. A. Segundo, S. Reis, J. Lima, *Anal. Chim. Acta*, **558**, 310–318 (2006).

Supplement

TABLE 1S. Number of Hydroxyl Groups and DPPH IC50 Values of Kaempferol, Quercetin, Baicalein, and Daidzein (Daidzein Was Measured Using ABTS•$^+$ method).

Flavonoids	*n*(OH)	IC50 (μM)
Kaempferol	4	16.11
Quercetin	5	4.94
Baicalein	3	11.36
Daidzein	2	–28.27 (for ABTS•$^+$)

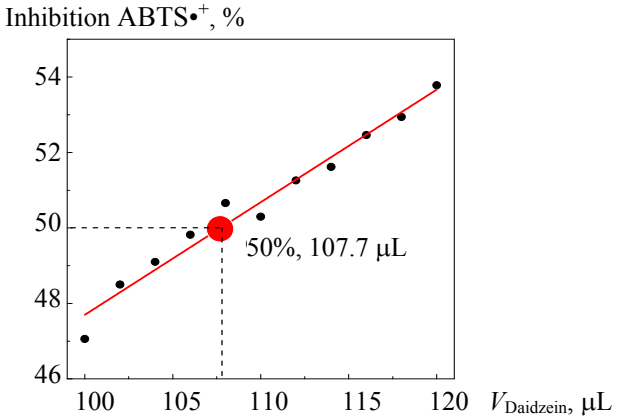

Fig. 1S. The added volumes of daidzein *vs.* the inhibition of ABTS•$^+$ radicals. The red dots mark the sample volumes with 50% inhibition, and the red line is the fitted line.

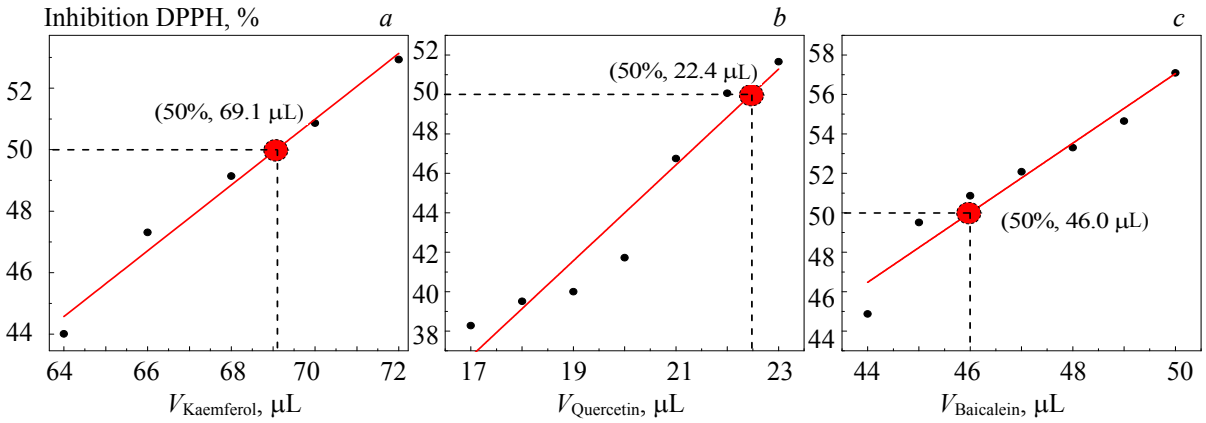

Fig. 2S. The added volumes of (a) kaempferol (0.1 mg/mL), (b) quercetin (0.1 mg/mL), and (c) baicalein (0.1 mg/mL) *vs.* the inhibition of DPPH radicals; the red dots mark the sample volumes with 50% inhibition, and the red line is the fitted line.

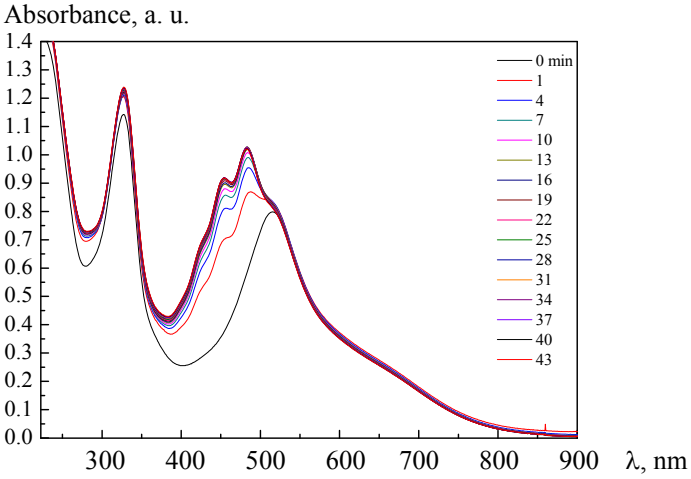

Fig. 3S. Steady-state absorption spectra of DPPH radical scavenging by the 6.21 μM β-carotene.