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Free Radical Scavenging Behavior of Antioxidant Compounds of Sesame (*Sesamum indicum* L.) in DPPH• System

KIZHIYEDATHU POLACHIRA SUJA, ANATHASANKARAN JAYALEKSHMY,* AND CHAMI ARUMUGHAN

Agroprocessing Division, Regional Research Laboratory (CSIR), Thiruvanathapuram 695019, India

The free radical scavenging capacity (RSC) of antioxidants from sesame cake extract was studied using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) on a kinetic model. Pure lignans and lignan glycosides isolated from methanolic extract by preparative HPLC were used in the study. To understand the kinetic behavior better and to determine the RSC of sesame antioxidants, the second-order rate constant (k_2) was calculated for the quenching reaction with [DPPH•] radical. The k_2 values of the sesame antioxidants were compared with those of butylated hydroxytoluene and α-tocopherol. The k_2 values for sesamol, sesamol dimer, sesamin, sesamolin, sesaminol triglucoside, and sesaminol diglucoside were 4.00×10^{-5}, 0.50×10^{-5}, 0.36×10^{-5}, 0.13×10^{-5}, 0.33×10^{-5}, and 0.08×10^{-5} μM^{-1} s^{-1}, respectively.

KEYWORDS: Sesame antioxidants; radical scavengers; DPPH• ; second-order kinetics

INTRODUCTION

Generation of active oxygen and free radicals is important both in food systems and in biological systems. In foods, the process of autoxidation and development of rancidity is caused by free radicals (*1*). Lipid peroxidation leads to the development of off-flavors and undesirable chemical compounds (*2*). In living systems, free radicals attack key biological molecules, leading to many degenerative disease conditions such as cancer, inflammation, atherosclerosis, and aging (*3*).

Recently, there has been growing interest in natural antioxidants of plant origin because they have greater application in the food industry for increasing the stability and shelf life of food products. Moreover, they also find use as nutraceuticals and phytoceuticals as they have significant impact on the status of human health and disease prevention (*4*). Natural antioxidants from dietary sources include polyphenols, carotenoids, vitamins, flavonoids, ascorbic acid, etc. Lignans are an important group of biologically active compounds of plant origin. They are known to possess a variety of biological activities, namely, antitumor, antimitotic, antiviral, and antiatherosclerotic activities (*5*).

Sesame is an important oilseed crop of the world, India being a major producer (*6*). Sesame seed provides a highly stable oil and nutritious protein and meal and is used in confectionery foods. It is also an ingredient in Ayurvedic oils under the Indian System of medicine. Sesame is reported to possess antiaging properties, hypocholesterolemic effect, alleviation of symptoms of alcohol withdrawal, etc. (*6*−*8*). Phytochemical investigation of sesame has revealed the presence of biologically active compounds, namely, lignans and lignan glucosides (*9*). Studies have shown that sesamin, the major compound present in sesame, inhibits cholesterol absorption and synthesis in rats (*10*). Other compounds such as sesamol and sesaminol are reported to be responsible for the increased stability of sesame oil (*11*). Recently, our investigation on natural antioxidants from industrial byproducts revealed the possibility of developing an antioxidant extract from sesame cake, and it is found to be effective in vegetable oil protection [details have been covered under a patent proposal, U.S. Appl. 60/404.004 (*12*)].

Lipid oxidation is a free radical mediated reaction. The antioxidant activity can be expressed in terms of radical scavenging ability during reaction with a specific radical such as [DPPH•] or [LOO•]. The [DPPH•] study is more widely quoted because the method can be followed more easily. If the reaction is followed kinetically, the rate at which the antioxidant reacts with radicals can be determined. There is no reported study on the radical scavenging capacity of individual antioxidant compounds from sesame seed/cake on a kinetic model. We have undertaken this investigation to examine the radical scavenging efficacy and, hence, the kinetic rate of reaction of individual compounds isolated from sesame cake extract in the [DPPH•] system.

MATERIALS AND METHODS

Materials. Sesame cake was obtained from local markets. Methanol was obtained from E. Merck, India. [DPPH•] was from Sigma Chemical Co. (St. Louis, MO). Sesamol dimer was prepared from standard sesamol according to the method of Kurechi et al. (*13*).

Preparation of Antioxidant Extract from Sesame Cake. Sesame cake was dried and powdered well. A 100 g sample was initially defatted with hexane in a Soxhlet extractor for 12 h. The defatted residue was further extracted with methanol for 16 h (Soxhlet extraction). The

* Address correspondence to this author at Natural Products, Organic Chemistry Division, Regional Research Laboratory (CSIR), Thiruvananthapuram 695019, Kerala, India [fax (0471) 2491712, 2490186; e-mail jayalekshmy_a@yahoo.com].

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extracts were filtered, solvent was removed under vaccum with a rotary evaporator (~45 °C), and the residue was redissolved in methanol and stored under refrigeration until further analysis.

HPLC Analysis. Identification and quantitation of lignans and lignan glucosides in sesame cake extract were carried out in a Shimazdu HPLC system with an LC-10AD model pump, a 7125 model Rheodyne injector fitted with a 20 μL sample loop, and an SPD-10A UV−visible detector, with a C-R7Ae plus integrator for data acquisition, analysis, and display. The sample was analyzed on a Waters μBondapak C_{18} column (4.5 mm i.d. × 25 cm) using a mobile phase of (70:30) methanol/water. Elution was performed at a flow rate of 1 mL/min, and the wavelength of detection was fixed at 290 nm.

Isolation and Characterization. The individual compounds were isolated in a Shimadzu (model LC-8A) preparative HPLC in an ODS column (Shimadzu, 250 × 20 mm i.d.) with the same conditions as for analytical HPLC. The fraction corresponding to each main peak was collected while eluting from the column and lyophilized to get pure compounds. For identification of compounds IR, NMR, and MS techniques were employed. IR spectra were recorded in Nicolet IR spectrometer. ^{1}H and ^{13}C NMR were taken in a Bruker Advance DPX_{300} series. MS spectra were recorded in a Shimadzu GCMS-QP 5050 series, in EI mode.

Free Radical Scavenging by DPPH•. [DPPH•] was dissolved in methanol (*14*, *15*), and the experiments were performed on freshly prepared solution. The assay conditions were as follows:

[DPPH•], 64 μM; and the isolated compounds sesamol, 40−320 μM; sesamol dimer, 145−2000 μM; sesamin, 1400−2900 μM; sesamolin, 270−1350 μM; sesaminol diglucoside, 140−870 μM; sesaminol triglucoside, 80−470 μM; butylated hydroxytoluene (BHT), 230−1700 μM; and α-tocopherol, 45−120 μM. Absorbance was taken at 515 nm during an interval from 10 s to 30 min of reaction in a UV−visible spectrophotometer (Shimadzu model 160A).

Kinetic Studies. Graphs and fittings of the experimental data were carried out in the Microcal Origin (version 6) program. The data points (180) of spectrophotometer readings of the disappearance of [DPPH•] in the presence of various antioxidant compounds were taken. The method of Espin et al. (*16*) who studied the radical scavenging capacity (RSC) of phenolic compounds by kinetic analysis, was followed in the present study. Second-order rate constants were calculated to measure the RSC of antioxidants. In this study, the decay of [DPPH•] from the medium has been assumed to follow pseudo-first-order kinetics, under the conditions of the reaction $[DPPH^\bullet]_0 \ll [AH]_0$, wherein one of the reactants is in large excess compared to the other and the concentration of the lesser component decreases exponentially. Under such conditions, the second-order reaction exhibits first-order characteristics and the reaction is studied as pseudo-first-order according to the fundamentals of chemical kinetics (*17*). The same assumption has also been made and reported by other researchers in similar [DPPH•] studies (*16*, *18*, *19*) and also according to equation

$$[DPPH^\bullet] = [DPPH^\bullet]_0 \, e^{-k_{obsd}t} \quad (1)$$

where [DPPH•] is the radical concentration at any time, $[DPPH^\bullet]_0$ is the radical concentration at time zero, and k_{obsd} is the pseudo-first-order rate constant.

[DPPH•] concentration in the reaction medium was calculated according to the method of Brand-Williams et al. (*14*) obtained from the calibration curve with the equation

$$A_{515nm} = 12509 \times [DPPH^\bullet] - 2.58 \times 10^{-3}$$

as determined by linear regression, where [DPPH•] is expressed as mol/dm^3.

From eq 1

$$\ln [DPPH^\bullet] = \ln [DPPH^\bullet]_0 - k_{obsd}t \quad (2)$$

the pseudo-first-order rate constant k_{obsd} was calculated. This constant was linearly dependent on the concentration of antioxidants, and from the slope of this plot, second-order rate constants (k_2) were calculated to determine the RSC of the different compounds (*16*, *18*, *19*).

Statistical Analysis. The results are expressed in terms of standard deviation for five independent determinations. Student's *t* test was applied to calculate the significant difference between values ($P \leq 0.05$).

RESULTS AND DISCUSSION

The kinetic behavior of the free radical scavenging compounds has been studied by allowing them to react with a stable free radical, namely, [DPPH•]. This assay was selected for this study because it measures the hydrogen-donating ability of antioxidants in a relatively short time compared to other methods and spectrophotometric characterization is also possible. This method is widely followed by many researchers (*14*−*16*). In the case of [LOO•] kinetics, the peroxide value is determined according to the active oxygen method in oil or a linoleic acid model system, and the method is time-consuming (*20*).

Sesame cake extract was analyzed by HPLC, and the peaks were initially identified by comparison of retention time (RT) values of sesamol (Sigma), sesamin, and sesamolin [isolated from sesame oil according to the method of Soliman et al. (*21*)]. Glucosides were initially compared for their sequence of elution by previous reports (*22*). The compounds present in sesame cake extract included sesamol, sesamin, sesamolin, sesaminol diglucoside, and sesaminol triglucoside. Pure compounds were isolated from defatted sesame cake extract by preparative HPLC, and their identification was confirmed by IR, NMR, and MS techniques; these spectroscopic data were compared with earlier reports (*23*, *24*). The spectroscopic data for glucosides were compared with those of Katsuzaki et al. (*24*). The purity of the compounds was checked by injecting them into an analytical HPLC to see whether the compound eluted as a single peak. In all of the above cases, compounds eluted as single peaks. The pure compounds, at various concentrations, were allowed to react with [DPPH•]. Sesamol dimer, which was detected in a few sesame extracts, was also included in the above study.

Espin et al. (*16*) studied the phenolic compounds and anthocyanins on the basis of second-order rate constants. Under the reaction conditions $[DPPH^\bullet] \ll [AH]$, the decay of [DPPH•] followed a pseudo-first-order rate equation. The pseudo-first-order rate constant k_{obsd} was linearly dependent on the concentration of antioxidants, and from the slope of this plot, the second-order rate constant could be calculated. Because the decay of [DPPH•] radicals by antioxidants in the present study also followed the same trend as that of anthocyanins, the above kinetic model study was found to be more suitable for the present study.

The scavenging reaction between [DPPH•] and antioxidant can be written as

$$[DPPH^\bullet] + (AH) \rightarrow DPPH{-}H + (A^\bullet) \quad (3)$$

(The new radical formed can undergo radical−radical interaction to render stable molecules

$$DPPH^\bullet + A^\bullet \rightarrow DPPH{-}A$$

$$A^\bullet + A^\bullet \rightarrow A{-}A$$

although these secondary reactions may be of limited occurrence (*25*, *26*).]

Absorbance decreases as the radical is scavenged by antioxidants through donation of hydrogen to form the reduced form DPPH−H, with the result the color changes from purple to yellow. The more rapidly the absorbance decreases, the more potent is the antioxidant compound in terms of hydrogen-donating ability.

In the presence of antioxidants, a decrease in the absorbance at 515 nm was measured until a steady state was observed. The reaction follows pseudo-first-order assay kinetics: $[DPPH^{\bullet}] \ll [AH]$.

Equation 3 becomes

$$-d[DPPH^{\bullet}]_t/dt = k_{obsd}[DPPH^{\bullet}]_t = k_2[(AH)][DPPH^{\bullet}]_t \quad (4)$$

The pseudo-first-order rate constant, k_{obsd}, when plotted against antioxidant concentration yielded a linear graph. From these plots, the second-order rate constant k_2 is calculated for the scavenging reaction of different antioxidants. This rate constant is related to the RSC of the antioxidants and is considered to be a measure of the rate of disappearance of [DPPH•]. The RSC values of the antioxidants from sesame were compared with those of α-tocopherol and BHT, which represent natural and synthetic antioxidants.

Accordingly, sesamol has the highest rate constant followed by α-tocopherol, sesamol dimer, BHT, sesamin, sesaminol triglucoside, sesamolin, and sesaminol diglucoside. The k_2 values for sesamol, sesamol dimer, sesamin, sesamolin, sesaminol triglucoside, and sesaminol diglucoside were 4.00×10^{-5}, 0.50×10^{-5}, 0.36×10^{-5}, 0.13×10^{-5}, 0.33×10^{-5}, and 0.08×10^{-5} μM^{-1} s^{-1}, respectively. The results showed significantly higher activity ($P \leq 0.05$) for sesamol and sesamol dimer compared to BHT. The activity of sesamol is significantly higher than that of tocopherol also.

There are many reports describing the antiradical effect of phenolic compounds by DPPH• assay (*14*, *15*), but the RSC of a compound varies depending on the assay method. Sanchez-Moreno (*15*) described a method to measure the antioxidant efficacy of polyphenols based on the EC_{50} (effective concentration of antioxidants required to scavenge 50% of the [DPPH•] radicals) and TEC_{50} (time to reach EC_{50}). Espin et al. calculated second-order rate constants to evaluate the RSC values of anthocyanins (*16*).

Very recently, a few preliminary studies on the antioxidant activity of ethanolic extracts of sesame coat have been studied by Chang et al. using the [DPPH•] assay. In this, the authors have reported the percentage activity of the extract to be less than those of α-tocopherol and BHA. The extract was found to contain sesamin and sesamolin (*27*). Similarly, Shyu et al. also studied the antioxidant activity of 80% methanolic extract of defatted sesame meal of Burma black sesame seeds by [DPPH•] assay (*22*). This crude extract was reported to contain sesaminol glucosides but not sesamol. The other compounds included in the above study were sesamin and sesamolin, and the percentage [DPPH•] scavenging power (after a fixed time) followed the order sesamin > sesamolin > sesaminol triglucoside > sesaminol diglucoside. The free radical scavenging activity of sesamol was compared with that of ascorbic acid by Kapadia et al. (*28*), and they have reported slightly higher IC_{50} values for sesamol. In all of these earlier studies the antioxidant activity is given as a percentage of [DPPH•] scavenging efficacy only, and data on the detailed kinetic analysis relating the effect of concentration or time with the antioxidant activity of the compounds are lacking. In the present study, the antiradical effectiveness of sesame antioxidants, namely, sesamol, lignans, and lignan glycosides isolated from sesame cake extract have been kinetically followed for the first time and second-order rate values calculated (**Table 1**). This study has been undertaken to get an idea about the rate at which the individual compounds scavenge the free radicals and also to know the contribution of each compound to the high antioxidant activity of sesame cake extract under study. The radical scavenging efficiency of sesamol dimer has not so far been studied by any method and is reported for the first time. In addition, the rate constants for the above compounds, namely, sesamol dimer, sesamin, sesamolin, sesaminol diglucoside, and sesaminol triglucoside, are reported for the first time. On the basis of the results of our study, all of these compounds possess radical scavenging activity toward [DPPH•], but to different degrees.

Table 1. Second-Order Rate Constants (k_2, μM^{-1} $s^{-1} \times 10^{-5}$) for the Reaction between DPPH• and Antioxidant Compounds

no.	antioxidant compound	$k_2 \pm$ SD
1	sesamol	4.00 ± 0.006
2	sesamol dimer	0.50 ± 0.015
3	sesamin	0.36 ± 0.010
4	sesamolin	0.13 ± 0.090
5	sesaminol triglucoside	0.33 ± 0.020
6	sesaminol diglucoside	0.08 ± 0.110
7	BHT	0.37 ± 0.009
8	α-tocopherol	3.71 ± 0.009

The presence of a methylene dioxy group is reported to be mainly responsible for the various biological activities of lignans (*5*). The stereochemistry of the furan−phenyl bond also contributes to the activity (*5*). In the case of sesamol and sesamol dimer, the presence of a hydroxyl group along with a methylene dioxy group may be responsible for the higher activity.

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