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Antioxidant Activity of Individual Steryl Ferulates from Various Cereal Grain Sources

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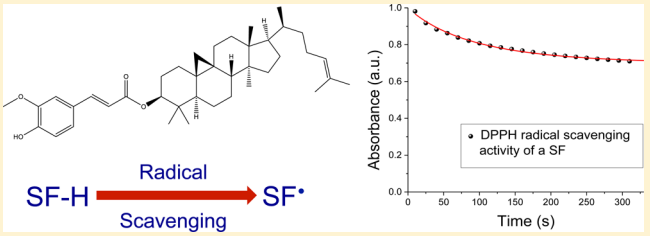
ABSTRACT: Steryl ferulates (SFs) are a subclass of bioactive lipids contributing to the health-promoting effects of whole grains. Most related studies focus on γ-oryzanol, a SF mixture from rice, since individual steryl ferulates are not commercially available. There is little evidence that individual SFs may vary in their bioactivity. The aim of this study was to evaluate the antioxidant activity of eight individual SFs by determining their radical scavenging capacity. Additional molecular properties of the individual SFs were determined by molecular simulation in order to identify correlations with their antioxidant activities. Our study demonstrates that individual SFs exhibit 1,1-diphenyl-2-picrylhydrazyl radical, hydroxyl radical, and superoxide anion radical scavenging abilities with subtle differences that were highly dependent on the kind of reaction taking place. The grouping of SFs by principle component analysis was mainly attributed to molecular properties, not antioxidant activities. Solvation energy was significantly correlated with some experimental observations. To our knowledge, this is the first study to evaluate the antioxidant activity of eight individual steryl ferulates from different sources. Results of this work will provide better insight into the antioxidant activity of SFs and the health benefits of whole grains.



Steryl ferulates (SFs) are the esters of phytosterols and ferulic acid, which are present in the bran of some grains such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), and triticale (×*Triticosecale Wittmack*).[1] They are bioactive lipids shown to possess health benefits such as lowering cholesterol,[2] inhibiting melanogenesis,[3] and exhibiting antioxidant[4] and anti-inflammatory[5] activities. To date, at least 21 different steryl ferulates, varying only in the type of sterol moiety, have been detected in various studies.[6−9] The different SFs investigated in this study are shown in Figure 1. Their total content, as well as the composition of individual SFs, varied depending on the grain source, genotype, and environmental factors.[10] In rice bran (total SFs, commonly known as γ-oryzanol (ORY), 1550−8400 $\mu g \cdot g^{-1}$ dry weight) the ratio of 4,4-dimethylsteryl ferulates (SFs **1** and **2**) and 4-desmethylsteryl ferulates (SFs **3**−**7**) is around 65:35.[11] However, only 4-desmethylsteryl ferulates are found in wheat bran (SFs **3**−**6**, total 297−584 $\mu g \cdot g^{-1}$) and corn bran (SFs **3**−**7**, total 200−250 $\mu g \cdot g^{-1}$).[7,12] SF **3** accounts for approximately 60% of SFs in wheat, while in corn, the most abundant is SF **4**, representing approximately 70% of total SFs.[1]

SFs have been shown to prevent oxidation in various biological systems. The mechanism of their antioxidant activity results from the phenolic proton in the ferulic acid moiety, which can be abstracted by any radical present in the media, and the resulting SF radical is stabilized by resonance along the π-electron system constituted by the aromatic ring and the carboxylate in *para* position to the phenol group.[7,13] Some researchers have also suggested that the SF radical might still influence oxidation, for example, by interfering with the chain reaction of lipid oxidation as alkyl radicals.[14] To date, bioactivity studies of SFs have mostly been performed with ORY due to the lack of individual SFs on the market. For instance, Kim et al. proved that ORY effectively improved flavor and oxidative stability of refrigerated cooked beef.[15] Juliano et al. demonstrated that ORY prevented AMVN-triggered lipoperoxidation and improved the oxidative stability of oils.[16] Recently, ORY was also determined to exhibit 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) and superoxide anion radical ($O_2^{\bullet-}$) scavenging activity, as well as a strong inhibition effect on linoleic acid peroxidation.[17]

Furthermore, there are indications that individual SFs may vary in their antioxidant activity, and therefore, the sterol composition may be an important aspect in defining the activity of SF mixtures. However, there are limited data available on these differences in antioxidant effects. Xu et al. reported that SFs **2** > **5** = **1** in preventing cholesterol oxidation.[18] Furthermore, in terms of preventing hydroperoxide formation in methyl linoleate bulk oil systems, the SF mixture from wheat and rye > SFs **6** and **8** > ORY and **1**.[4] Huang demonstrated that the antioxidant activity of SF **2** > **1** and SF **5** > ORY in an SVEC-10 mouse lymph endothelial cell model, using tert-butyl hydroperoxide (tBHP) as an oxidizing agent.[19] Since their antioxidant capacities differ considerably from one to another, it is of great interest to evaluate the activities of individual SFs more systematically.

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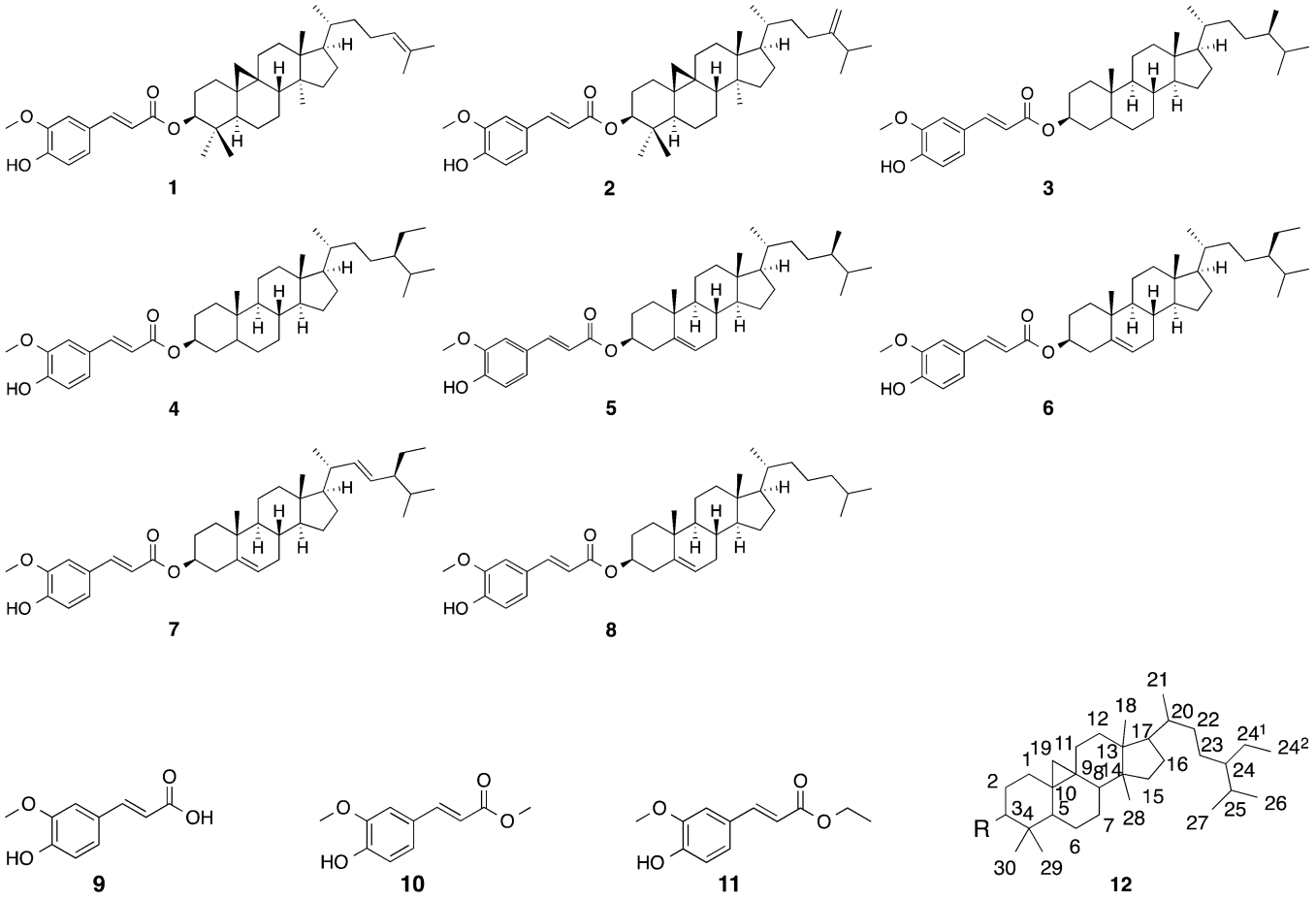

Figure 1. Molecular structures of (**1**) cycloartenyl ferulate, (**2**) 24-methylenecycloartanyl ferulate, (**3**) campestanyl ferulate, (**4**) sitostanyl ferulate, (**5**) campesteryl ferulate, (**6**) sitosteryl ferulate, (**7**) stigmasteryl ferulate, (**8**) cholesteryl ferulate, (**9**) ferulic acid, (**10**) methyl ferulate, (**11**) ethyl ferulate, and (**12**) a general sterol skeleton based on IUPAC-IUB 1989.

Table 1. Kinetic Parameters for the DPPH Radical Reaction[a]

		absorbance ($t \rightarrow \infty$)		
compound	k ($\mu M^{-1}\cdot s^{-1}$)	1.67 μM	16.7 μM	60 μM
1	0.010 ± 0.004	1.301 ± 0.001	1.109 ± 0.003	0.72 ± 0.01
2	0.011 ± 0.002	1.283 ± 0.001	1.030 ± 0.004	0.82 ± 0.01
3	0.018 ± 0.004	1.198 ± 0.001	1.091 ± 0.005	0.79 ± 0.03
4	0.015 ± 0.002	1.156 ± 0.001	0.991 ± 0.004	0.64 ± 0.01
5	0.013 ± 0.002	1.273 ± 0.001	1.045 ± 0.004	0.70 ± 0.01
6	0.013 ± 0.001	1.298 ± 0.001	0.944 ± 0.004	0.64 ± 0.01
7	0.012 ± 0.002	1.144 ± 0.001	1.010 ± 0.004	0.72 ± 0.01
8	0.012 ± 0.002	1.245 ± 0.001	0.979 ± 0.005	0.74 ± 0.01
9	0.012 ± 0.001	1.232 ± 0.001	0.913 ± 0.005	0.53 ± 0.01
10	0.014 ± 0.002	1.008 ± 0.001	0.896 ± 0.005	0.57 ± 0.01
11	0.013 ± 0.002	0.992 ± 0.001	0.851 ± 0.003	0.58 ± 0.01
ORY	0.011 ± 0.006	1.246 ± 0.001	0.981 ± 0.003	0.69 ± 0.01
WB	0.014 ± 0.002	1.245 ± 0.001	0.992 ± 0.006	0.65 ± 0.01
pyrogallol	0.12 ± 0.01			

[a]Data are expressed as mean ± SEM (n = 3–9).

The aim of this study was to evaluate the antioxidant activity of eight individual SFs. SFs **2**–**6** were purified from ORY and wheat bran. SF **7** and SF **8**, which occur only in trace amounts in nature, were synthesized. SFs were divided into groups according to structure similarity: SFs **1** and **2** (with methyl groups at C4 and C14 and a cyclopropyl ring at C9/C10); SFs **3** and **4** (with a saturated sterol, no double bond at C5 and C6); and SFs **5**, **6**, **7**, and **8** (with unsaturated sterol and a double bond at C5 and C6). Additionally, antioxidant effects of ferulic acid (**9**), methyl ferulate (**10**), ethyl ferulate (**11**), ORY, and the SF mixtures from wheat bran (WB) were evaluated. Antioxidant activity was determined by evaluating the scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (DPPH$^{\bullet}$) in methanol, hydroxyl radical ($^{\bullet}$OH), and

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superoxide anion radical ($O_2^{\bullet -}$) in an *in vitro* water environment. Additional molecular properties of individual SFs were determined by molecular simulation, in order to correlate them with their antioxidant activities. As far as we know, this study is the first to provide a comprehensive comparison of the antioxidant activity of individual SFs.

RESULTS AND DISCUSSION

Scavenging Effect on DPPH Radical. DPPH$^\bullet$ scavenging measurement was carried out with antioxidants at concentrations of 1.67, 16.7, and 60.0 μM. The irreversible reaction is presented by eq 1. The decrease of the concentration of DPPH$^\bullet$ ([DPPH$^\bullet$]) over time can be written as shown in eq 2, following global second-order kinetics. In the reaction system, the concentrations of DPPH$^\bullet$ and product $DPPH_2$ can be determined from eqs 3 and 4 with a rate constant k, as well as initial concentrations of DPPH$^\bullet$ ($[DPPH^\bullet]_0$) and SF ($[SF]_0$). The kinetics parameters, k value, and plateau value (the absorption when $t \rightarrow \infty$) were obtained from curve-fitting of the absorbance vs time plots (Table 1).

$$DPPH^\bullet + SF \xrightarrow{k} DPPH_2 + SF^\bullet \quad (1)$$

$$-\frac{d[DPPH^\bullet]}{dt} = k[DPPH^\bullet][SF] \quad (2)$$

$$[DPPH^\bullet] = [DPPH^\bullet]_0 \left(1 - \frac{1 - e^{([DPPH^\bullet]_0 - [SF]_0)kt}}{1 - \frac{[DPPH^\bullet]_0}{[SF]_0} e^{([DPPH^\bullet]_0 - [SF]_0)kt}} \right) \quad (3)$$

$$[DPPH_2] = [DPPH^\bullet]_0 \left(\frac{1 - e^{([DPPH^\bullet]_0 - [SF]_0)kt}}{1 - \frac{[DPPH^\bullet]_0}{[SF]_0} e^{([DPPH^\bullet]_0 - [SF]_0)kt}} \right) \quad (4)$$

All of these antioxidants showed DPPH$^\bullet$ scavenging effect. Each antioxidant showed the same rate constant at different concentrations, confirming the kinetic order of the scavenging process. Rate constants of individual SFs decreased in the following order: SFs **3** and **4** ≥ SFs **5**, **6**, **7**, and **8** ≥ SFs **1** and **2**. ORY, which contains mainly SFs **1** and **2**, had a low rate constant, but with high experimental error. Additionally, WB was found to show a higher rate constant than ORY, which was in agreement with the behavior of individual SFs. Furthermore, ferulates **10** and **11** had moderate and similar kinetics. However, compound **9**, the smallest molecule in this study, did not exhibit any advantage in the scavenging process. Moreover, the positive control molecule, pyrogallol, was observed to have a kinetic constant nearly 10-fold of that of the ferulates and compound **9**. Nevertheless, the differences in rate constants among ferulates and compound **9** in this reaction were very small.

The absorbance in the final state or plateau ($t \rightarrow \infty$; A_∞) was also obtained from the curve-fitting. Moreover, the efficiency (ε) of each antioxidant on DPPH$^\bullet$ scavenging can be determined from

$$\varepsilon_{DPPH} = \frac{A_{0(DPPH)} - A_{\infty(DPPH)}}{A_{0(DPPH)}[SF]_0} \quad (5)$$

where $A_{0(DPPH)}$ is the absorbance of DPPH$^\bullet$ solution without any antioxidant and $A_{\infty(DPPH)}$ is the absorbance in the presence of an antioxidant when the reaction is finished ($t \rightarrow \infty$). Generally, the efficiencies of ferulates, as well as compound **9**, decreased with increasing concentrations (Figure 2). This could

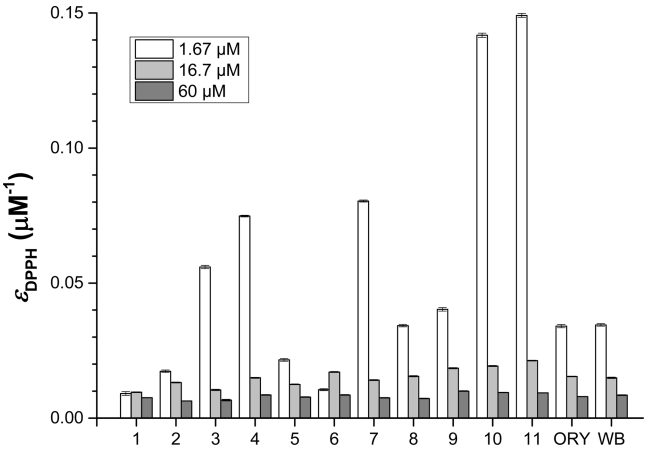

Figure 2. Efficiency of DPPH radical scavenging.

be explained by the lower relative amount of DPPH$^\bullet$ per unit of antioxidant in treatments with higher concentrations of antioxidant, based on the constant concentration of DPPH$^\bullet$. At the lowest antioxidant concentration of 1.67 μM, the differences in DPPH$^\bullet$ scavenging efficiency among these compounds were very clear. Ferulates **10** and **11** were observed to have the highest efficiency. This may be partially explained by the fast diffusion of such small molecules compared to the SFs ($D = k_B T/6\pi\eta R$, where k_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, and R is the radius of the solute molecule). Ferulates **10** and **11** have much smaller radii than SF molecules; hence they had higher diffusion coefficients (D) and exhibited higher reaction efficiencies. However, compound **9**, which also has the smallest radius of molecules studied, showed only a moderate efficiency, which could be explained by the strong solvent interaction between the polar protic solvent (methanol) and the carboxylic group of compound **9**, especially when compound **9** was at very low concentrations. Additionally, compound **9** may form noncovalent intermolecular interactions and dimerize via O–H···O hydrogen bonds between carboxylic groups, which may further restrict its diffusion compared to the small ferulates. High efficiencies were demonstrated by SFs **7**, **3**, and **4**, as compared with other SFs; however, according to the results of the simulation studies, SFs **7**, **3**, and **4** did not have the smallest radii. We therefore infer that other factors in the reaction may contribute to this observation, for example intra- or intermolecular interactions or solvent interaction. When the concentration of antioxidant was increased to 16.7 and 60 μM, the differences in efficiencies among ferulates and compound **9** were less notable. Ferulates **10** and **11** and compound **9** showed slightly higher efficiencies than the SFs at 16.7 μM; nevertheless, all the compounds had similar efficiencies at 60 μM. This suggests that when the concentration of antioxidant was very low, the number of effective collisions was greatly influenced by the solvent effect; meanwhile with higher concentrations of antioxidant, the effective collisions could mostly result from its high relative concentration.

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The DPPH• scavenging activities of SFs have also been reported in previous studies. Akiyama et al. reported similar DPPH• scavenging activity for SFs **1**, **2**, **5**, and **6** and compound **9** (>100 μM).[20] Islam et al., who used a comparable reaction system with ours, also found SFs **1**, **2**, and **6** and compound **9** had similar DPPH• scavenging activities.[21] In another study, Kikuzaki et al. reported that SF **1**, SF **2**, and ORY (20 μM) had the same DPPH• scavenging capacity, while compound **9** was slightly better than the SFs.[22] Moreover, compound **9** has also been reported to be a better DPPH• scavenger than SFs **6** and **8**, as well as the SF mixture from rye and wheat (17 μM).[4] However, all of these studies only compared scavenging capacities after the reaction. To the best of our knowledge, ours is the first study to investigate reaction kinetics with DPPH•, which provide more information on individual SFs as DPPH• scavengers.

Scavenging of Hydroxyl Radical. In our study, •OH scavenging capacity was determined using electron spin resonance (ESR) with the spin trap DMPO method. The •OH is very short-lived, and DMPO is commonly used to trap •OH, as the DMPO-OH adduct has a half-life of 12−156 min in neutral solutions.[23] Due to the limitation of solubility, the highest concentration of SF studied was 15.0 μM. Generally, all the compounds in this study scavenged the •OH concentration dependently (Table 2). At 1.5 μM, similar •OH scavenging capacities (19−25%) were observed among the antioxidants tested. At 2.5 μM, ferulate **10** (29%) was only slightly more effective than SF **1** and WB (21% and 22%, respectively), and other compounds did not differ from each other. While using a higher concentration of 5 μM, ferulates **10** and **11** as well as SF **2** exhibited higher scavenging capacities (around 36%) than SF **1** (27%). Nevertheless, at the highest concentration of 15.0 μM, all the ferulates, as well as compound **9**, showed very similar activities (39−44%). In this study, compound **9**, which has a higher solubility than the ferulates, did not show any advantage in scavenging •OH. In this water environment (pH around 7.4), the carboxyl group of compound **9** could be deprotonated, leaving only the proton from the phenolic hydroxyl group available for •OH scavenging, which could explain why the stable, deprotonated form (carboxylate) may exhibit similar behavior to other ferulates in this reaction system.

Table 2. Hydroxyl Radical Scavenging Activity (RSA)[a]

	RSA (%)			
compound	1.5 μM	2.5 μM	5.0 μM	15.0 μM
1	23 ± 2	21 ± 2	27 ± 5	43 ± 3
2	19 ± 3	24 ± 1	36 ± 3	45 ± 4
3	23 ± 2	27 ± 2	34 ± 2	43 ± 2
4	22 ± 3	25 ± 4	28 ± 2	40 ± 2
5	21 ± 3	25 ± 3	29 ± 2	39 ± 3
6	20 ± 3	24 ± 3	31 ± 2	43 ± 1
7	23 ± 4	26 ± 4	30 ± 1	44 ± 2
8	24 ± 3	24 ± 3	30 ± 3	40 ± 3
9	23 ± 3	26 ± 1	33 ± 3	40 ± 3
10	19 ± 5	31 ± 2	36 ± 1	43 ± 3
11	19 ± 6	29 ± 4	36 ± 3	42 ± 3
ORY	25 ± 1	25 ± 4	31 ± 3	42 ± 3
WB	23 ± 2	22 ± 1	33 ± 3	43 ± 1

[a]Data are expressed as mean ± SD (n = 3).

Islam et al. also reported SFs **1**, **2**, and **6** (concentration of 40 μM, with 0.1% supporting solvent ethanol: DMSO 9:1) had similar •OH scavenging capacities, while compound **9** showed significantly higher activity than the SFs.[21] However, Juliano et al. reported that ORY (concentrations of 1.65 and 16.5 μM, with 1% ethanol) had no •OH scavenging activity, when measured by its inhibition of *p*-nitrosodimethylaniline-trapping of •OH.[16] Given the differences in the reaction systems, our data are not directly comparable with their findings. Generally, all the antioxidants in our study were found to have similar •OH scavenging abilities.

Scavenging of Superoxide Anion Radical. Scavenging of $O_2^{\bullet-}$ was investigated with antioxidants at 40, 80, and 160 μM. This method examined the ability of antioxidants to compete with the probe nitrotetrazolium blue chloride (NBT) in scavenging $O_2^{\bullet-}$. The irreversible reaction of NBT with $O_2^{\bullet-}$ in the system is described by eq 6. At the onset of the reaction, the concentration of $O_2^{\bullet-}$ ($[O_2^{\bullet-}]_0$) is much higher than that of NBT ($[NBT]_0$); then the decrease in the concentration of NBT ([NBT]) over time can be written, as shown in eq 7, as a pseudo-first-order kinetic process. Furthermore, the concentration of NBT and the corresponding product $NBTH_2$ can be determined from eqs 8 and 9 with rate constant k_1 and $[NBT]_0$ as parameters. The reaction of SF with $O_2^{\bullet-}$ in the system has the same kinetics as NBT, as shown in eqs 10 through 13. The kinetic parameter, k_1, of reaction 6 and the absorbance of $NBTH_2$ when the reaction is finished ($t \rightarrow \infty$; A_∞) were also obtained from the curve-fitting experimental measurements (Table 3). The differences in k_1 and A_∞ reflect the competitive ability of SFs in reacting $O_2^{\bullet-}$ with NBT.

$$2O_2^{\bullet-} + NBT \xrightarrow{k_1} 2O_2 + NBTH_2 \quad (6)$$

$$-\frac{d[NBT]}{dt} = k_1[O_2^{\bullet-}]^2[NBT] \approx k_1[NBT] \quad (7)$$

$$[NBT] = [NBT]_0 e^{-k_1 t} \quad (8)$$

$$[NBTH_2] = [NBT]_0(1 - e^{-k_1 t}) \quad (9)$$

$$O_2^{\bullet-} + SF \xrightarrow{k_2} O_2 + SF^{\bullet} \quad (10)$$

$$-\frac{d[SF]}{dt} = k_2[O_2^{\bullet-}][SF] \approx k_2[SF] \quad (11)$$

$$[SF] = [SF]_0 e^{-k_2 t} \quad (12)$$

$$[SF^{\bullet}] = [SF]_0(1 - e^{-k_2 t}) \quad (13)$$

As shown in Table 3, some of the rate constants of NBT were lower with higher concentrations of antioxidant (SFs **2**, **5**, **6**, **7**, **8**), suggesting these compounds interfere with NBT dose dependently. The rate constant was dramatically lower (40%) in the presence of SFs **7** and **8** at concentrations of 80 and 160 μM. Meanwhile, for SFs **5** and **6**, significantly lower rate constants (30%) were observed only with the high concentration of 160 μM. Moreover, for SF **2**, the effect was gradual: 0%, 30%, and 45%, for concentrations of 40, 80, and 160 μM, respectively. In the case of SF **1**, which has a similar structure to SF **2**, the rate was consistently low (30%) compared to the NBT reaction at all three concentrations. Furthermore, SFs **3** and **4** demonstrated interference with the NBT reaction (by approximately 30%) only at concentrations of 40 and 160 μM. On the other hand, for the SF mixtures, WB and ORY were

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Table 3. Kinetic Parameters for Superoxide Anion Radical Reaction[a]

	k_1 (min^{-1})			absorbance ($t \rightarrow \infty$)		
compound	40 μM	80 μM	160 μM	40 μM	80 μM	160 μM
1	0.12 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	1.8 ± 0.1	1.57 ± 0.06	1.48 ± 0.06
2	0.18 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	1.68 ± 0.05	1.79 ± 0.09	1.80 ± 0.06
3	0.13 ± 0.03	0.19 ± 0.01	0.13 ± 0.01	1.89 ± 0.01	1.47 ± 0.03	1.57 ± 0.08
4	0.14 ± 0.02	0.21 ± 0.01	0.14 ± 0.01	1.85 ± 0.05	1.35 ± 0.02	1.47 ± 0.05
5	0.17 ± 0.01	0.18 ± 0.01	0.13 ± 0.01	1.65 ± 0.04	1.40 ± 0.03	1.19 ± 0.05
6	0.19 ± 0.01	0.19 ± 0.01	0.13 ± 0.01	1.64 ± 0.06	1.38 ± 0.04	1.16 ± 0.03
7	0.18 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	1.68 ± 0.05	1.76 ± 0.07	1.78 ± 0.06
8	0.17 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	1.76 ± 0.04	1.71 ± 0.08	1.82 ± 0.08
9	0.20 ± 0.01	0.14 ± 0.03	0.19 ± 0.02	1.27 ± 0.07	1.4 ± 0.2	1.03 ± 0.08
10	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.02	1.90 ± 0.01	1.72 ± 0.06	1.6 ± 0.2
11	0.14 ± 0.01	0.16 ± 0.03	0.13 ± 0.02	1.90 ± 0.01	1.7 ± 0.1	1.6 ± 0.2
ORY	0.17 ± 0.01	0.15 ± 0.04	0.18 ± 0.01	1.60 ± 0.04	1.4 ± 0.2	1.33 ± 0.07
WB	0.21 ± 0.01	0.10 ± 0.01	0.20 ± 0.01	1.52 ± 0.03	1.75 ± 0.09	1.19 ± 0.02
NBT alone	0.188 ± 0.001			1.90 ± 0.01		

[a]Data are expressed as mean ± SEM (n = 3).

able to interfere with NBT only at a concentration of 80 μM, with approximately 45% reduction in the rate constant for WB and 20% for ORY. Additionally, both ferulates **10** and **11** exhibited a 20% difference in rate constants from the NBT reaction for all tested concentrations. However, compound **9** only slightly interfered with the NBT reaction at 80 μM. Nevertheless, SFs were able to effectively compete with NBT-trapping of $O_2^{\bullet-}$. SFs **2**, **7**, and **8** appeared to have slightly higher competitive abilities than the other individual SFs, especially at the highest concentrations.

The absorbance of $NBTH_2$ when the reaction was completed ($t \rightarrow \infty$; A_∞) was also obtained from curve-fitting (Table 3), to determine the amount of $O_2^{\bullet-}$ in the system. The efficiency (ε) of each antioxidant in reducing $O_2^{\bullet-}$ can be determined from eq 14:

$$\varepsilon_{O_2^{\bullet-}} = \frac{A_{0(NBTH_2)} - A_{\infty(NBTH_2)}}{A_{0(NBTH_2)}[SF]_0} \quad (14)$$

where $A_{0(NBTH2)}$ is the initial absorbance of $NBTH_2$ without the presence of antioxidant, and $A_{\infty(NBTH2)}$ is the absorbance of $NBTH_2$ after complete reaction with the antioxidant. The efficiencies of most of the antioxidants in this study were lower with higher concentrations (Figure 3). At the 40 μM level, the differences in efficiency were very clear among these compounds. Compound **9**, which is the most water-soluble antioxidant at pH 7.4, was found to have the highest efficiency. Among the SFs, the SF mixture WB was the most effective, followed by ORY. SFs **2**, **5**, **6**, and **7** showed similar efficiency and were slightly more effective than SFs **1** and **8**. Furthermore, the weakest SFs were found to be SFs **3** and **4**. However, ferulates **10** and **11**, which consistently decreased the rate constant of NBT by 20%, cannot scavenge $O_2^{\bullet-}$ at this concentration. From this, we deduce that ferulates could be considered as amphiphile molecules due to the hydrophilic (phenolic hydroxyl group) and lipophilic (methyl, ethyl, or sterol moiety) characteristics, and therefore self-assembly may occur in this environment. Their different potentials to aggregate in the water/solvent mixture may induce their different efficiencies on radical scavenging. When applying a higher concentration of 80 μM, all the antioxidants showed $O_2^{\bullet-}$ scavenging activity. Compound **9**, ORY, and SFs **3**–**6** were observed to have similar efficiency and were slightly more effective than the other compounds. Moreover, ferulates **10** and **11** showed relatively low efficiency at scavenging $O_2^{\bullet-}$. At the concentration of 160 μM, the highest efficiency was still obtained with compound **9**, followed by SFs **5** and **6**, ORY, and WB. Overall, among all individual SFs, SFs **5** and **6** generally showed the highest efficiency to scavenge $O_2^{\bullet-}$ at all tested concentrations.

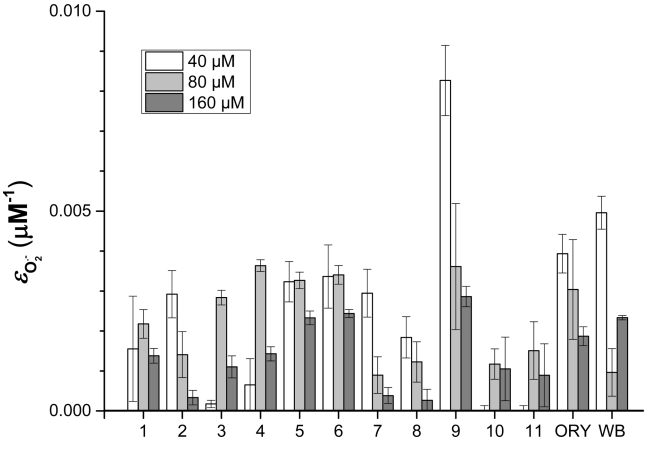

Figure 3. Efficiency of superoxide anion radical scavenging.

However, the $O_2^{\bullet-}$ scavenging effect of SFs was not observed by Juliano et al.[16] They reported that ORY at the concentration of 10 μM had no scavenging activity in a system where $O_2^{\bullet-}$ was produced by spontaneous autoxidation of $FeCl_2$ in morpholinepropanesulfonic acid buffer. Recently, Saenjum et al. reported that ORY showed an $O_2^{\bullet-}$ scavenging effect (IC_{50} 30 μM) in a phenazine methosulfate–β-nicotinamide adenine dinucleotide system.[17] Due to the different radical-generating systems and concentrations of antioxidants employed, our data are not directly comparable with their findings. To the best of our knowledge, this is the first study to investigate and compare the $O_2^{\bullet-}$ scavenging activity of individual SFs.

Simulation and Correlation Analysis. We performed molecular simulations to explain the differences in antioxidant activity from the point of view of the SF's molecular properties. The dipole moment and quantitative structure–activity relationship parameters, i.e., log$P$, surface area, volume, and solvation energy (hydration), were obtained from the quantum

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Table 4. Molecular Properties from Molecular Simulations

compound	Connolly surface area (Å^2)	Connolly volume (Å^3)	solvation energy (kcal·mol^{-1})	log*P*	polarizability (Å^3)	dipole moment (D)	molar mass (Da)
1	928	1734	−4.31	7.72	71	3.55	602.9
2	943	1776	−4.60	8.09	72	3.72	616.9
3	914	1705	−3.92	7.61	68	3.55	578.9
4	939	1754	−3.72	8.01	70	3.52	592.9
5	924	1706	−4.15	7.17	68	3.51	576.9
6	945	1748	−3.98	7.57	70	3.49	590.9
7	942	1745	−4.35	7.31	69	3.45	588.9
8	919	1672	−4.28	6.84	66	3.49	562.8
9	379	591	−14.1	−0.63	20	4.07	194.2
10	414	652	−8.77	−0.60	21	3.74	208.2
11	450	709	−8.03	−0.25	23	3.73	222.2

mechanics simulations (Table 4). Among the individual SFs, SF **8** was found to have the smallest volume, as well as the smallest log*P* value, while SF **2** had the highest dipole moment and solvation energy. Generally, the differences between SFs with respect to these parameters were very small.

The grouping of SFs was performed by principal component analysis (PCA) with variables of their antioxidant activities and molecular properties (Figure 4), but compounds **9**−**11** were not included for further analyses. The overview of SFs **1**−**8**, as well as all the variables, is presented in a bi-plot (Figure 4A). PC1 and PC2 explained 94% and 4% (in total 98%) of the variance. Considering the PCA scores (Figure 4B), four groups could be proposed, namely, SFs **1** and **2**, SFs **4**, **6**, and **7**, SFs **3** and **5**, and SF **8**. Correlation loadings (Figure 4C) showed that the molecular properties, except for solvation energy, had a position at the right side of PC1, close to the 100% explained variance circle, indicating that these variables greatly contribute to the SF groupings. However, the variables related to the SF's antioxidant activities were located near the inner ellipse (less than 50% of explained variance), indicating these variables had little influence. Nevertheless, the variable of solvation energy was very close to the position of antioxidant activity, suggesting they are somehow correlated.

Pearson's correlation was performed for compounds **1**−**8** to support the correlation loadings of PCA; compounds **9**−**11** are not shown because they do not fall in the same linear tendency. Examining all the correlations between antioxidant activities and molecular properties of the eight SFs led us to propose that solvation energy was more likely to influence antioxidant activity (Figure 5), and the trend was that the higher the solvation energy, the better the antioxidant activity. Higher solvation energy—less released energy when solvent molecules bond to the SF surface—led to less solvation of the SF molecule due to the number of established bonds and the distance/angle between the solvent and the substrate. Furthermore, SF species were available to react with radicals in the system. With respect to molecular properties, solvation energy was identified as a significantly correlated parameter to the scavenging properties only in some observations.

Previous studies also reported that different SFs, as well as compound **9**, vary in antioxidant activity. SFs were observed to show less antioxidant activity than compound **9** in some models, e.g., scavenging DPPH$^{\bullet}$[4,22] or ABTS$^{\bullet+}$,[24] which was primarily explained by solubility or steric hindrance. However, SFs were also found to be better antioxidants than compound **9** in scavenging oxygen radical and in a cooked meat model system.[25] In some studies, 4-desmethylsteryl ferulates were observed to be better antioxidants than 4,4-dimethylsteryl ferulates in DPPH$^{\bullet}$[4] and oil models,[4,24,26] for which it was suggested to be a relic of the negative effects from the dimethyl groups at C4 as well as the cyclopropyl ring at C9/C19. Moreover, saturated 4-desmethylsteryl ferulates were observed to have slightly higher antioxidant activity during frying than those with a double bond at C5/C6.[24] Nevertheless, some observations also showed similar antioxidant activity for individual SFs in DPPH.[20,21] In this study, generally, the difference in antioxidant activity of individual SFs was very small and depended greatly on the reaction models. In addition to the inner-molecular variations, the intermolecular interactions, as well as interactions with the solvent (environment), should also be considered. Thus, the same SF compound might show different scavenging properties depending on the substrate studied, as well as the kind of radical scavenging process taking place. Therefore, the antioxidant activity of SFs should be reconsidered based on the reaction occurring, and with respect to the food product to be studied, along with other synergistic processes that might happen in natural settings.

EXPERIMENTAL SECTION

General Experimental Procedures. Acetic acid (Ph Eur) and 1-butanol (Ph Eur) were purchased from Merck, Darmstadt, Germany. Acetone (≥99.9%), acetonitrile (ACN; ≥99.9%), cholesterol (99%), 3,4-dihydro-2*H*-pyran (97%), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; ≥99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid dipotassium salt dihydrate (EDTA; ≥99.0%), hydrochloric acid (37%), hydrogen peroxide solution (H_2O_2; ≥35%), hypoxanthine (HPX; ≥99%), iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$; ≥99%), methanol (≥99.9%), nitrotetrazolium blue chloride (NBT; 98%), pyrogallol (≥99%), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; ≥99%), *p*-toluenesulfonic acid monohydrate (≥98%), *trans*-ferulic acid (≥99%), and xanthine oxidase (XOD) from bovine milk (Grade IV) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Stigmasterol was bought from Research Plus, Bayonne, NJ, USA. Cycloartenyl ferulate (≥99%; SF **1**) was purchased from Wako, Osaka, Japan. γ-Oryzanol was obtained from CTC Organics, Atlanta, GA, USA. Methyl ferulate (99%) and ethyl ferulate (99%) were from Alfa Aesar, Ward Hill, MA, USA. Wheat bran was obtained from a commercial milling of a mixture of wheat varieties from Swissmill, Switzerland.

Preparative HPLC (Merck-Hitachi, Japan) with an XBridge Prep Shield RP C_{18} column (5.0 μm, 10 × 250 mm, Waters, Ireland), analytical HPLC (Agilent 1100, Germany) with an XBridge Shield C_{18} column (3.5 μm, 3 × 150 mm, Waters, Ireland), a U-2800 UV/vis spectrophotometer (Hitachi, Japan), and a MiniScope MS300 benchtop electron spin resonance spectrometer (ESR) (Magnettech, Berlin, Germany) were used for analyses. ^{1}H NMR experiments were carried out on a Bruker Avance spectrometer (Bruker BioSpin GmbH,

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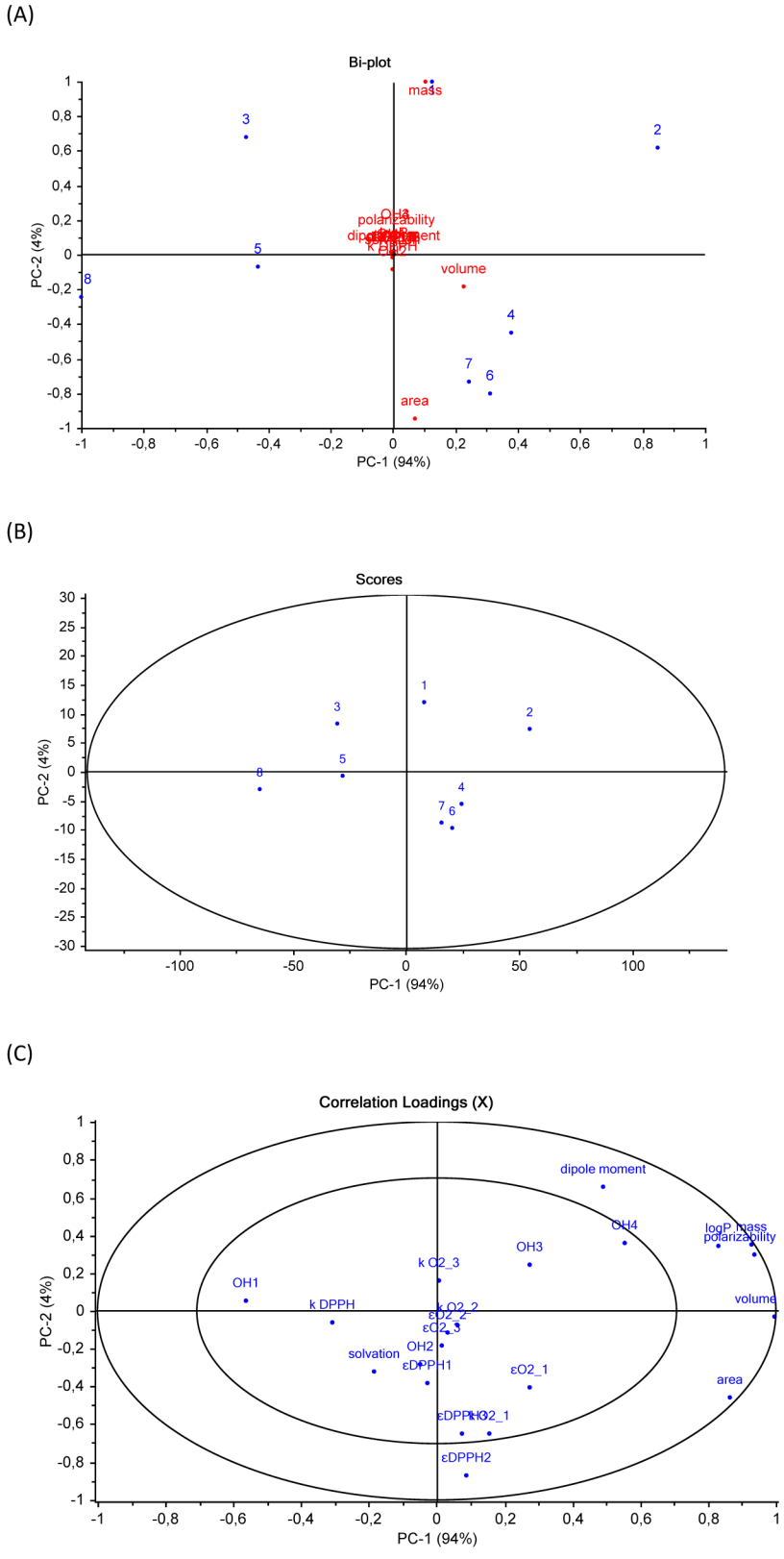

Figure 4. Principal component analysis (PCA) of antioxidant activity and molecular properties for the individual SFs (**1**–**8**). (A) Bi-plot. (B) Scores plot of SFs with Hotelling's T^2 ellipse at 5% of confidence. (C) Loadings plot of the variables: (a) antioxidant properties: k_{DPPH}; ε_{DPPH} at 1.67, 16.7, and 60 μM (εDPPH1, εDPPH2, and εDPPH3, respectively); $k_{O2}^{\bullet-}$ at 40, 80, and 160 μM (k O2_1, k O2_2, and k O2_3, respectively); $\varepsilon_{O2}^{\bullet-}$ at 40, 80, and 160 μM (εO2_1, εO2_2, and εO2_3, respectively); $^{\bullet}$OH scavenging activity at 1.5, 2.5, 5.0, and 15.0 μM (OH1, OH2, OH3, and OH4, respectively); (b) molecular properties: area, volume, polarizability, dipole moment, solvation, log*P*, and molar mass).

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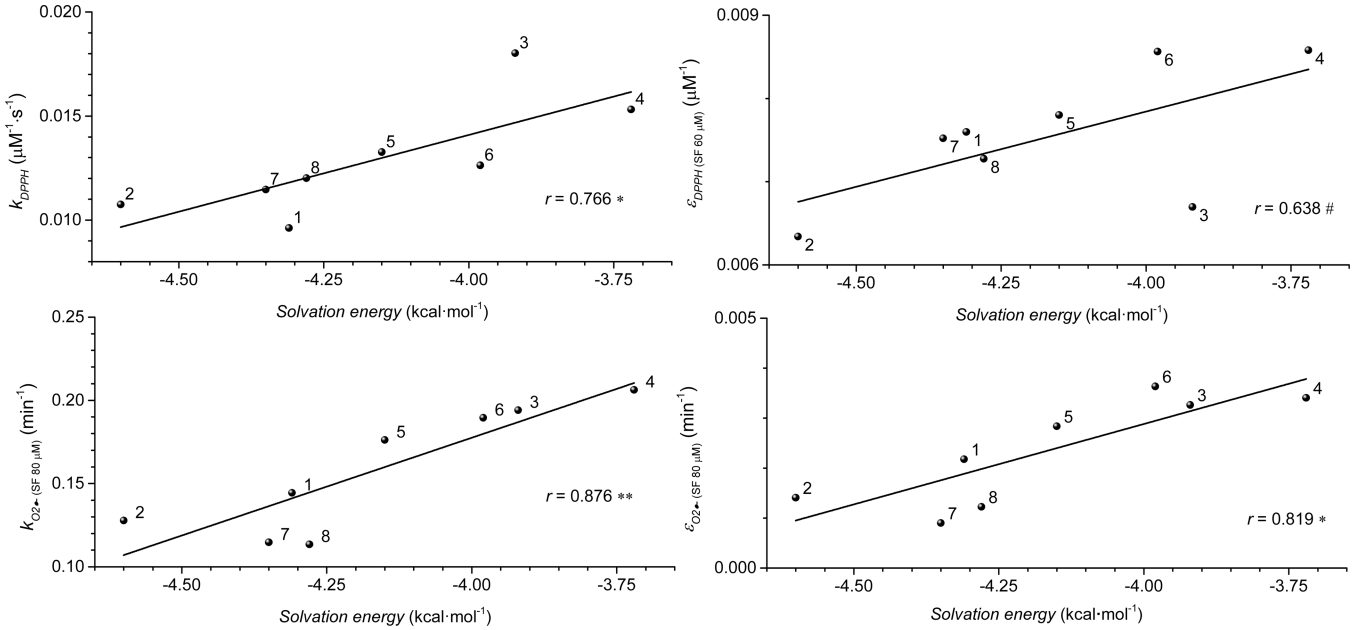

Figure 5. Linear correlation among antioxidant activities (*y*-axis) and solvation energy (*x*-axis) of individual SFs (**1**–**8**) based on Pearson's correlation coefficients (*r*). The symbol *: the correlation is significant at the 0.05 level (2-tailed); **: significant at the 0.01 level (2-tailed); #: significant at the 0.05 level (1-tailed).

Rheinstetten, Germany) operating at 400 MHz (^{1}H) and using $CDCl_3$ as solvent and as internal standard.

Synthesis of SF 7 and SF 8. A new two-step synthetic approach was designed for the synthesis of high-purity SFs **7** and **8**. The first step was Steglich esterification using the protected tetrahydropyranyl ferulic acid and stigmasterol or cholesterol. The molecules obtained were the corresponding protected SF **7** and **8** derivatives, for which good yields (85%) were isolated. The second and final step was cleavage of the previous protected molecules, carried out in the presence of methanol and catalytic amounts of *p*-toluenesulfonic acid, yielding the target molecules with very good yields (>99%). Both molecules were analyzed by NMR and HPLC-MS to confirm high purity of the compounds synthesized according to the starting stigmasterol and cholesterol.

SF 7: ^{1}H NMR (400 Hz, $CDCl_3$, δ) 7.59 (1H, d, ArCH═, J = 15.0 Hz), 7.05 (2H, m, Ar), 6.90 (1H, d, Ar, J = 7.8 Hz), 6.27 (1H, d, OCOCH═, J = 15.0 Hz), 5.83 (1H, s, OH), 5.40 (1H, t, CH═, J = 6.3 Hz), 5.15 (1H, dd, CH═, J = 15.2 Hz, J = 8.5 Hz), 5.01 (1H, dd, CH═, J = 15.1 Hz, J = 8.5 Hz), 4.74 (1H, m, CHOCO), 3.92 (3H, s, OCH_3), 2.39 (2H, d, $OCHCH_2C$═, J = 3.1 Hz), 2.1–0.8 (38H, stigmasterol), 0.70 (3H, s, CH_3) ppm.

SF 8: ^{1}H NMR (400 Hz, $CDCl_3$, δ) 7.59 (1H, d, ArCH═, J = 15.0 Hz), 7.05 (2H, m, Ar), 6.90 (1H, d, Ar, J = 7.8 Hz), 6.27 (1H, d, OCOCH═, J = 15.0 Hz), 5.85 (1H, s, OH), 5.40 (1H, t, CH═, J = 6.3 Hz), 4.74 (1H, m, CHOCO), 3.92 (3H, s, OCH_3), 2.39 (2H, d, $OCHCH_2C$═, J = 3.1 Hz), 2.1–0.8 (38H, cholesterol), 0.68 (3H, s, CH_3) ppm.

Extraction and Purification of SFs 2–6. Extraction and purification of SFs **2**–**6** from ORY and wheat bran was performed by the method we previously designed and reported.[27] First, total lipids from wheat bran were extracted at 50 °C with acetone, then subjected to base–acid cleanup in order to eliminate neutral lipids. Subsequently, the residues from wheat bran and ORY were purified by preparative HPLC using a UV detector at 325 nm and ACN–H_2O–butanol–acetic acid (88:6:4:2, v/v/v/v) as eluent, with a 6.6 mL·min^{-1} flow rate at 25 °C. SF **2** was collected only from ORY. WB and SFs **3** and **4** were collected from the wheat bran. SFs **5** and **6** were collected from both ORY and wheat bran. Purity and quantification of SFs were also carried out with an analytical HPLC using a UV detector at 325 nm, and MS was used to confirm the high purity of the compounds according to the method previously reported.[28] For quantification, SF **1**, the only commercially available SF standard, was used as an external standard.

Eight SFs in total were used in this study: SFs **1**, **2**, **3**, **4**, **5**, **6**, **7**, and **8**. The respective purities, reported as area percentage from the HPLC analysis, were 99%, 96%, 95%, 98%, 98%, 96%, 99%, and 97%, and the purity of the WB and ORY were 99% and 95%, respectively.

DPPH Radical Scavenging Activity Measurement. The DPPH$^\bullet$ scavenging activity method used in this study was modified from Nyström et al.[4] The DPPH solution was freshly prepared in methanol and was brought to a concentration of 112 μM in the reaction system. The compounds **1**–**11**, ORY, and WB were also dissolved in methanol with final concentrations of 1.7, 16.7, and 60.0 μM, respectively, in the reaction system. Pyrogallol in methanol (final concentration 66 μM) was used as the positive control. After mixing the antioxidant and DPPH for 10 s, the absorbance at 517 nm was recorded immediately and subsequently every 15 s for 5 min. Reaction kinetics were analyzed by fitting the absorbance vs time curves with Origin 9.0.

Hydroxyl Radical Scavenging Activity Measurement. The $^\bullet$OH scavenging activity method used in this study was a modified version of the methods reported by Cheng et al.[29] and Faure et al.[23] The $^\bullet$OH radical was generated by the Fenton reaction and measured with the spin-trapping technique by ESR. The solutions were added in the following order: 30 μL of 250 mM spin trap DMPO, 30 μL of 1.0 mM H_2O_2, 30 μL of 1.0 mM EDTA, 22.5 μL of H_2O, 7.5 μL of antioxidants in ACN at various concentrations or ACN alone as control, and 30 μL of 1.0 mM $FeSO_4$ to initiate the reaction. The solutions of DMPO, H_2O_2, EDTA, and $FeSO_4$ were prepared in Milli-Q water. Compounds **1**–**11**, ORY, and WB were each dissolved in ACN at four concentrations, 0.03, 0.05, 0.1, and 0.3 mM, thus leading to final concentrations of 1.5, 2.5, 5.0, and 15.0 μM in the respective reaction systems. An aliquot of the reaction mixture was loaded in a 50 μL micropipet, and the ESR spectra were recorded. The ESR parameters were as follows: B0-field, 3350 G; sweep width, 100 G; steps, 4096; sweep time, 30 s; number of passes, 2; modulation frequency, 1000 mG; microwave attenuation, 10 dB; and receiver gain, 900. TEMPO in H_2O (2 μM) was used as a daily reference standard for the ESR instrument. The relative ESR signal was obtained by calculating the ratio of the peak-to-peak amplitude of the second singlet in the ESR signal of DMPO and the peak-to-peak amplitude of the first singlet in the ESR signal of the TEMPO. The preparation and

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measurement were operated at room temperature. The percentage of radical scavenging activity was calculated by RSA% = $(h_0 - h_x)/h_0 \times 100$, where h_0 is the ESR signal in the control and h_x is the relative ESR signal of the antioxidants.

Superoxide Anion Radical Scavenging Activity Measurement. The $O_2^{\bullet-}$ scavenging activity method applied was modified from Saint-Cricq de Gaulejac et al.[30] and Zhou et al.[31] The $O_2^{\bullet-}$ was generated by the enzymatic hypoxanthine−xanthine oxidase (HPX-XOD) system. The molecular probe NBT reacted with $O_2^{\bullet-}$, forming formazan ($NBTH_2$), which could be detected at 560 nm. This method measures the ability of antioxidants to compete with NBT in scavenging $O_2^{\bullet-}$. In this method, 2 mM HPX, 0.56 U·mL^{-1} XOD, and 0.34 mM NBT were prepared in 50 mM phosphate buffer (PBS) at pH 7.4. The blank solution contained 300 μL of PBS, 200 μL of NBT, and 500 μL of HPX. The solutions required for the reaction were added in the following order: 200 μL of NBT, 500 μL of HPX, 50 μL of PBS, 50 μL of antioxidants in DMSO or DMSO alone for control, and 200 μL of XOD to initiate the reaction. Compounds **1**−**11**, ORY, and WB were each dissolved in DMSO at concentrations of 0.8, 1.6, and 3.2 mM, leading to 40, 80, and 160 μM in the final systems, respectively. The absorbance at 560 nm was recorded every minute once the reaction started, for a total of 7 min. Reaction kinetics were analyzed by fitting the absorbance vs time curves with Origin 9.0.

Simulation. Structures were drawn using HyperChem 8.0.3 software, and the semiempirical quantum mechanics method Austin Model 1 (AM1), together with the Polak−Ribiere conjugate gradient algorithm with a root-mean-square (RMS) gradient of 0.05 kcal·Å^{-1}·mol^{-1}, was used for the geometrical optimization of the molecules. The spin-pairing restricted Hartree−Fock (RHF) operators were used for neutral species, while unrestricted Hartree−Fock (UHF) operators were employed for radicals. The self-consistent field (SCF) convergence limit was set at 10^{-5}, and the accelerated convergence procedure was used. The dipole moment and some quantitative structure−activity relationship (QSAR) parameters were obtained from the quantum mechanics simulations. The Connolly surface area and solvation energy (hydration) were evaluated using the TIP3P water model, where the dimension of the solvent molecule was set to 1.4 Å radius.

Data Analysis. Principle component analysis was conducted with Unscrambler X 10.1 (Oslo, Norway) to obtain a complete view of comparisons of individual SFs with their antioxidant activities, as well as molecular properties. The relationships of variables were also measured using Pearson's correlation coefficients with IBM SPSS Statistics 19.0 (Armonk, NY, USA).

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Notes

The authors declare no competing financial interest.

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