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Evaluation of the Antioxidant Activity of Capsiate Analogues in Polar, Nonpolar, and Micellar Media

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Synthesis of 10 capsiate analogues was conducted by lipase-mediated (Novozyme 435) esterification of vanillyl alcohol with different fatty acids. The antioxidant activity of the synthesized capsiates was evaluated using three in vitro assays: DPPH radical scavenging assay (polar medium), Rancimat assay (nonpolar medium), and autoxidation of linoleic acid (micellar medium). The objective of this study is to find the influence of structural characteristics of the alkyl chain of capsiate analogues on their antioxidant activity. In these assays, BHT and α-tocopherol were used as reference compounds. Both DPPH and Rancimat assays did not show any specific trend of antioxidant activity with the increase in lipophilicity and also with the type of fatty acids grafted to the phenolic moiety. In the Tween 20 micellar system for the inhibition of autoxidation of linoleic acid, vanillyl ester attached to a C18 alkyl chain (vanillyl stearate, oleate, and ricinoleate) exhibited maximum inhibition of autoxidation of linoleic acid.

KEYWORDS: Antioxidant; autoxidation of linoleic acid; capsaiciate; capsiate; Rancimat assay; DPPH assay

INTRODUCTION

Antioxidants are classified as free radical scavengers and are important in human health. Due to exposure to radiation and several other oxidative stresses, reactive oxygen species (ROS) are generated in living organisms. ROS are responsible for the oxidative modification of cell membranes, triggering altered cellular mechanisms (*1*−*3*). Fatty acids, especially polyunsaturated fatty acids (PUFAs), are most susceptible to attack by ROS. Moreover, due to the prevalence of PUFAs in the neuronal system, ROS are particularly active in the brain and neuronal tissue, causing many neurodegenerative diseases (*4*). Although the endogenous antioxidant defense mechanism in living organisms prevents such oxidative damages, any imbalance in this defense mechanism may lead to overproduction of oxidants. Under such circumstances, exogenous antioxidants such as dietary supplements are considered to be a promising approach in the prevention and treatment of diseases caused by oxidants. On the other hand, antioxidants are also important for cosmetic and food products. Lipid oxidation not only reduces the nutritive value of the food products but also affects the sensory properties and shelf life (*5*). For decades antioxidants have been used as food preservatives to control deterioration in the form of oxidation. Some of the well-known natural antioxidants used by the food industry are α-tocopherols, vitamin C, and carotenoids. Natural phenolic compounds such as flavonoids, polyphenols, and phenolic acids have also recently gained interest as food preservatives (*6*).

Capsaicinoids and capsinoids are two families of naturally occurring lipophilic alkaloids derived from peppers (*Capsicum annuum* L.). Capsaicinoids are responsible for the pungent spicy flavor of peppers (*7*, *8*). These are reported to possess many potent bioactivities, such as anti-inflammatory, antimicrobial, antimutagenic, and antitumor properties (*9*, *10*). They are also used as topical analgesics for treating pain and for the enhancement of thermogenesis and fat consumption in mammals (*8*, *11*). Even though capsaicinoids show interesting antioxidant properties, pungency and obnoxious properties limit their application as food-grade antioxidants. On the other hand, capsinoids are nonpungent ester analogues of capsaicinoids and are known to share many potent biological activities of capsaicinoids. Notable among them are suppression of fat accumulation, induction of apoptosis, and anticancer and antioxidant properties (*6*, *8*). Because of their nonpungency, capsinoids are more palatable than capsaicinoids and may find application in the food and beverage industry as useful ingredients. Capsinoids are structurally similar to capsaicinoids, the only difference being an ester bond connecting the hydrophobic chain to the phenol moiety instead of an amide bond as in capsaicinoids. Capsinoids have been isolated from the fruit of sweet pepper cultivar, CH-19 Sweet (*12*). The most naturally abundant capsinoid is capsiate (4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate). Other capsinoids such as dihydrocapsiate (4-hydroxy-3-methoxybenzyl 8-methyl-6-nonanoate) and nordihydrocapsiate (4-hydroxy-3-methoxybenzyl 7-methyloctanoate) have also been isolated (*13*).

The limited availability of natural capsinoids and their complicated and costly chemical synthesis have restricted their applications in the food and cosmetic industries. This has necessitated the synthesis of their linear analogues. There are few reports on the synthesis of capsiates and their analogues following chemical as well as enzymatic approaches (*10*, *14*, *15*). In the present work, the synthesis of a series of capsiate analogues was conducted by lipase-mediated (Novozyme 435) esterification

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of vanillyl alcohol with different fatty acids. It is well-known that the polarity of the environment strongly governs the antioxidant activity of phenolic antioxidants (*16*). Accordingly, the antioxidant activity of all the synthesized capsiate analogues was assayed in three different media, namely, polar, nonpolar, and micellar media. These assays are the diphenylpicrylhydrazine (DPPH) radical scavenging assay (polar medium), the Rancimat assay (nonpolar medium), and autoxidation of linoleic acid (micellar medium). The nature and type of alkyl chain grafted to the phenolic moiety are often used as tools to change the physical properties of the antioxidants. The objective of this study is to find the influence of change in structural feature of alkyl chain of capsiate analogues, especially variation in chain length, and also inclusion of a functional group in the hydrophobic chain on their antioxidant activity in these three in vitro assays.

MATERIALS AND METHODS

General Experimental Procedures. The synthesized capsiate analogues were purified by silica gel (60−120 mesh) column chromatography (Acme Synthetic Chemicals, Mumbai, India) and identified by thin-layer chromatography (TLC), FT-IR, MS, and NMR analyses. TLC was performed on precoated silica gel 60 F_{254} from Merck (Darmstadt, Germany). All ^{1}H and ^{13}C NMR spectra were recorded on 300 and 75 MHz (Varian, Palo Alto, CA) spectrometers, respectively. Mass spectra were recorded either on a VG Auto Spec-M (Manchester, U.K.) or on an Agilent 5973 mass spectrometer (Palo Alto, CA) in the EI mode and are given in mass units (m/z). A λ-35 UV−vis spectrophotometer from Perkin-Elmer (Shelton, CT) was used in the scavenging assays and also for the estimation of conjugated diene during autoxidation of linoleic acid. The oxidative stability of soybean oil was measured in a Metrohm A.G. (model 743) Rancimat apparatus (Herisau, Switzerland).

Chemicals. Vanillyl alcohol, DPPH radical, and fatty acids, such as octanoic, undecanoic, 10-undecenoic, dodecanoic, hexadecanoic, octadecanoic, and octadec-9-enoic acids, were purchased from Fluka (Buchs, Switzerland). Erucic and behenic acids were purchased from M/s VVF Limited (Mumbai, India). Ricinoleic acid was isolated from castor oil and purified by column chromatography in the laboratory. Linoleic acid (≥99%) and α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO). Immobilized lipase from *Candida antarctica* (Novozym 435) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Refined soybean oil, purchased from the local market, has the following fatty acid composition (in wt %), as determined by GC: palmitic acid, 11.3%; stearic acid, 5.2%; oleic acid, 22.4%; linoleic acid, 52.6%; linolenic acid, 6.1%; arachic acid, 0.9%; behenic acid, 1.2%; and lignoceric acid, 0.3%. The rest of the chemicals and solvents were purchased from SD-Fine Chem (Mumbai, India).

General Procedure for the Synthesis of Capsiate Analogues. Syntheses of capsiate analogues were carried out following the method reported by Kobata et al. (*15*) with little modification. Briefly, equimolar concentrations of vanillyl alcohol and fatty acid were solubilized in *tert*-butanol followed by the addition of Novozyme 435 (5% by wt of total substrates). The reaction mixture was stirred at 55 °C, and the progress of the reaction was monitored by TLC (eluant/30% ethyl acetate in hexane). After maximum conversion (4 h), the reaction mixture was filtered to separate the lipase, and the lipase was washed with ethyl acetate. The filtrate was washed with saturated sodium bicarbonate and water. The organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using hexane/ethyl acetate (96:4 v/v) to elute capsiate analogues. Isolated yields of these synthesized compounds are in the range of 55−72%. Structural confirmation of all the synthesized compounds were conducted by NMR, IR, and mass spectroscopy and matched well with those reported in the literature (*10*, *15*, *17*). The spectral data of four new analogues are given below.

4-Hydroxy-3-methoxybenzyl undecenoate (***c***): ^{1}H NMR ($CDCl_3$, 300 MHz), δ 1.28 (m, 10H, alkyl −C$\underline{H}_2$), 1.61 (m, 2H, −CO—CH_2—C$\underline{H}_2$), 2.02 (m, 2H, C$\underline{H}_2$—CH=CH_2), 2.29 (t, 2H, J = 7.554 Hz), 3.91 (s, 3H, −OC$\underline{H}_3$), 4.88−5.01 (dd, 2H, CH=C$\underline{H}_2$ and s, 2H, −OC$\underline{H}_2$Ph), 5.50 (s, 1H, Ph—O$\underline{H}$), 5.67−5.83 (m, 1H), 6.80−6.85 (m, 3H, aromatic); ^{13}C NMR ($CDCl_3$, 75 MHz), δ 173.7 (−C$\underline{O}$−), 146.4, 145.6, 127.6, 121.7, 114.3, 111.1 (aromatic), 138.8 (C$\underline{H}$=CH_2), 113.9 (CH=C$\underline{H}_2$), 66.1 (−O$\underline{C}H_2$Ph), 55.6 (−O$\underline{C}H_3$), 34.1, 33.5, 29.0, 28.9, 28.8, 28.6, 24.7 (alkyl $\underline{C}H_2$); HRMS calcd for $C_{19}H_{28}O_4Na$ [M + Na]$^+$, 343.1885; found, 343.1895.

4-Hydroxy-3-methoxybenzyl behenate (***h***): ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.88 (t, 3H, −CH_2−C$\underline{H}_3$, J = 6.798 Hz), 1.25 (m, 36H, alkyl −C$\underline{H}_2$), 1.61(m, 2H, −CO—CH_2—C$\underline{H}_2$) 2.29 (t, 2H, −CO—C$\underline{H}_2$−, J = 7.554 Hz), 3.90 (s, 3H, −OC$\underline{H}_3$), 4.99 (s, 2H, −OC$\underline{H}_2$Ph), 5.52 (s, 1H, Ph—O$\underline{H}$), 6.80−6.85 (m, 3H, aromatic); ^{13}C NMR ($CDCl_3$, 75 MHz), δ 173.7 (−C$\underline{O}$−), 146.4, 145.7, 127.9, 121.9, 114.3, 111.2 (aromatic), 66.2 (−O$\underline{C}H_2$Ph), 55.8 (−O$\underline{C}H_3$), 34.3, 31.8, 29.7, 29.5, 29.4, 29.2, 29.0, 27.1, 24.9, 22.6 (alkyl −C$\underline{H}_2$−), 14.0 (−CH_2—C$\underline{H}_3$); HRMS calcd for $C_{30}H_{52}O_4Na$ [M + Na]$^+$, 459.3763; found, 459.3767.

4-Hydroxy-3-methoxybenzyl erucate (***i***): ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.89 (t, 3H, −CH_2−C$\underline{H}_3$, J = 6.798 Hz), 1.26 (m, 32H, alkyl −C$\underline{H}_2$), 1.61 (m, 2H, −CO—CH_2—C$\underline{H}_2$), 2.0 (m, 4H, −C$\underline{H}_2$—CH=CH—C$\underline{H}_2$−), 2.30 (t, 2H, −CO—C$\underline{H}_2$−, J = 7.554 Hz), 3.91 (s, 3H, −OC$\underline{H}_3$), 4.98 (s, 2H, −OC$\underline{H}_2$Ph), 5.29 (m, 2H, −C$\underline{H}$=C$\underline{H}$−), 5.50 (s, 1H, Ph—O$\underline{H}$), 6.79−6.88 (m, 3H, aromatic); ^{13}C NMR ($CDCl_3$, 75 MHz), δ 173.7 (−C$\underline{O}$−), 146.4, 145.7, 127.9, 121.9, 114.3, 111.2 (aromatic), 129.8 (−C$\underline{H}$=C$\underline{H}$−), 66.2 (−O$\underline{C}H_2$Ph), 55.8 (−O$\underline{C}H_3$), 34.3, 31.8, 29.7, 29.5, 29.4, 29.2, 29.0, 27.1, 24.9, 22.6 (alkyl −C$\underline{H}_2$−), 14.0 (−CH_2—C$\underline{H}_3$); HRMS calcd for $C_{30}H_{50}O_4Na$ [M + Na]$^+$, 497.3606; found, 497.3614.

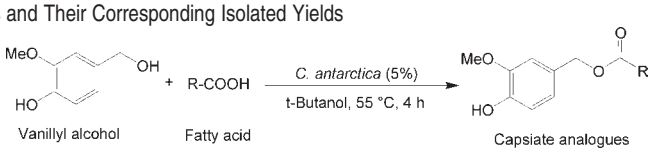
4-Hydroxy-3-methoxybenzyl ricinoleate (***j***): ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.89 (t, 3H, −CH_2—C$\underline{H}_3$, J = 6.987 Hz), 1.28−1.45 (m, 18H, alkyl −C$\underline{H}_2$), 1.60 (m, 2H, −CO—CH_2—C$\underline{H}_2$), 1.97−2.07 (m, 2H, CH_2—C$\underline{H}_2$—CH=CH), 2.17 (t, 2H, CH=CH—C$\underline{H}_2$, J = 6.610 Hz), 2.29 (t, 2H, −CO—C$\underline{H}_2$, J = 7.365 Hz), 3.56 (m, 1H, (−CH_2(HO)C$\underline{H}$—CH_2−), 3.89 (s, 3H, −OC$\underline{H}_3$), 4.98 (s, 2H, −OC$\underline{H}_2$Ph), 5.22−5.41 (m, 1H, CH=C$\underline{H}$−), 5.43−5.58 (m, 1H, −C$\underline{H}$=CH−), 6.79−6.86 (m, 3H, aromatic); ^{13}C NMR ($CDCl_3$, 75 MHz), δ 173.7 (−C$\underline{O}$−), 146.4, 145.7, 127.9, 125.1, 121.9, 114.3, 111.2 (aromatic), 133.3 (−C$\underline{H}$=CH−), 71.5 (−CH_2(HO)C$\underline{H}$—CH_2−), 66.2 (−O$\underline{C}H_2$Ph), 55.8 (−O$\underline{C}H_3$), 36.8, 35.3, 34.3, 31.8, 29.5, 29.3, 29.0, 27.3, 25.6, 24.9, 22.5 (alkyl—C$\underline{H}_2$−), 14.0 (−CH_2—C$\underline{H}_3$); HRMS calcd for $C_{26}H_{42}O_5Na$ [M + Na]$^+$, 457.2929; found, 457.2949.

Antioxidant Activity Assays. *DPPH Radical Scavenging Assay*. The antioxidant activity was determined by the radical scavenging ability using the stable DPPH radical (*18*). Briefly, 200 μL of methanolic solution of the synthesized capsiate analogues (1 mM) was added to 2 mL of methanolic solution of DPPH radical (0.1 mM), and the total volume was made up to 3 mL with methanol. After 40 min of incubation at 30 °C in the dark, the absorbance of the mixture was measured at 517 nm against methanol as blank. α-Tocopherol and BHT were used as positive controls, and their concentrations were kept the same as that of the synthesized analogues. The free radical scavenging activity (FRSA in %) of the tested samples was evaluated by comparison with a control (2 mL of DPPH radical solution and 1 mL of methanol). Each sample was measured in triplicate and averaged. The FRSA was calculated using the following formula: FRSA = $[(A_c - A_s)/A_c] \times 100$, where A_c is the absorbance of the control and A_s is the absorbance of the tested sample after 40 min.

Rancimat Assay. The antioxidant activity of the synthesized capsiate derivatives in a food matrix was also performed with soybean oil by the well-established Rancimat method (*19*). The Rancimat apparatus was operated at 110 °C with a dry air flow of 20 L/h, passed through the oil sample (5 ± 0.001 g) containing a 1 mM concentration of the reference antioxidants (BHT and α-tocopherol) or synthesized capsiate analogues. All tests were performed in triplicate using soybean oil of one batch to avoid batch-to-batch variation. The volatile oxidation products generated during the oxidation of the oil caused an increase in the electrical conductivity of the water. The time until there was a sharp increase of conductivity value, corresponding to the inflection point of the curve, was considered to be the induction time, expressed in hours. An increase in the induction time indicates increased antioxidant potency of the compound added to the soybean oil.

Autoxidation of Linoleic Acid. The rate of inhibition of autoxidation of linoleic acid in micelle by antioxidant was measured according to the method of Chimi et al. (*20*), with some modifications. Initially, phosphate buffer (pH 6.9) containing 0.5% Tween 20 was prepared. Linoleic acid (2.5×10^{-3} M) was dispersed in the above buffer along with 1 mM concentrations of reference antioxidants (BHT and α-tocopherol) or synthesized capsiate analogues. Samples were left in the dark and in air for 5 days at 50 °C. Samples containing linoleic acid but without antioxidants (reference and

Table 1. Synthesized Capsiate Analogues and Their Corresponding Isolated Yields



capsiate analogue	R	isolated yield (%)
a	$-(CH_2)_6CH_3$	72
b	$-(CH_2)_9CH_3$	70
c	$-(CH_2)_8—CH=CH_2$	71.4
d	$-(CH_2)_{10}CH_3$	71
e	$-(CH_2)_{14}CH_3$	65.7
f	$-(CH_2)_{16}CH_3$	70
g	$-(CH_2)_7CH=CH—(CH_2)_7CH_3$	67.4
h	$-(CH_2)_{20}CH_3$	55
i	$-(CH_2)_{11}CH=CH—(CH_2)_7CH_3$	61.3
j	$-(CH_2)_7CH=CH—CH_2—CH(OH)—(CH_2)_5CH_3$	58.4

synthesized capsiates) and the controls (containing 1 mM concentrations of corresponding antioxidants) without linoleic acid were also incubated under the same conditions. The autoxidation of linoleic acid is accompanied by the generation of conjugated diene, which was measured by UV at 234 nm. Samples were diluted 20 times with phosphate buffer containing 0.5% Tween 20 before measuring the absorbance. A decrease in the rate of formation of conjugated diene indicates the increased antioxidant activity of the compound added to the micelle of linoleic acid.

Statistical Analysis. All of the assay results reported in the present work are the mean of three measurements (presented as mean ± SD) and were analyzed by a paired Student's *t* test to evaluate the level of statistical significance. Differences were assessed by one-way analysis of variance. A p value of < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Phenolic lipids are one of the most well-studied classes of natural antioxidants. This is due to their many potent additional biological activities. Both capsaicinoids and capsinoids belong to this class of antioxidant. However, capsinoids assume significance as exogenous antioxidant compared to capsaicinoids for their application as dietary supplements and also as preservatives in cosmetic and food products. This is due to the fact that capsinoids are nonpungent ester analogues of capsaicinoids having very similar biological activities (*6*). However, synthesis of natural capsinoids is complicated and costly due to the very specific nature of the alkyl side chain. To have commercial application in the food and cosmetic industries, it is necessary to synthesize capsiate analogues with linear alkyl chain and evaluate their antioxidant potency. In the present work, easily available natural fatty acids are selected for the synthesis of capsiate analogues.

Reports on the synthesis of capsiate analogues are very few in the literature (*10*, *12*, *14*, *15*). Kobata et al. (*12*) first reported the chemical synthesis of capsiate, but due to poor yield as well as lengthy workup, the same authors reported lipase-catalyzed synthesis of vanillyl nonanoate, the straight-chain analogue of natural capsiate (*15*). Appendino et al. (*14*) reported a chemoselective Mitsunobu esterification of vanillyl alcohol with nonanoic acid. In the present work, lipase-catalyzed esterification of vanillyl alcohol with different fatty acids was carried out for the synthesis of capsiates. Altogether 10 compounds were synthesized by varying the alkyl chain length as well as the functionality in the alkyl chain (**Table 1**). Equimolar concentrations of vanillyl alcohol and fatty acid were esterified using the *Candida antartica* lipase (Novozyme 435) at 50 °C for 4 h. After completion of reaction, the lipase was filtered, and the synthesized compounds were purified by column chromatography. The protocol is simple and regioselective and affords excellent isolated yield (55−72%).

Table 2. DPPH Radical Scavenging Activity of the Synthesized Capsiate Analogues and Reference Antioxidants

capsiate analogue[a]	free radical scavenging activity[b] (FRSA) (%)
a	77.98 ± 1.14 ∗
b	78.88 ± 1.03 ∗
c	78.76 ± 1.56 †
d	76.91 ± 0.81 ∗
e	70.17 ± 0.99 ∗,§
f	66.65 ± 1.23 ∗,§
g	67.57 ± 0.16 ∗,§
h	59.74 ± 0.75 ∗,§
i	61.76 ± 3.11 ∗,§
j	86.8 ± 2.78 §
BHT	87.51 ± 0.90 ∗,†
α-tocopherol	79.01 ± 0.86 §

[a] Refer to **Table 1** for **a** −**j**. [b] Values are mean ± SD (n = 3). FRSA was significantly different versus BHT as shown by ∗, $p < 0.001$, and †, $p < 0.01$. FRSA was significantly different versus α-tocopherol as shown by §, $p < 0.001$.

The antioxidant activity of all the synthesized compounds was determined by the radical scavenging ability using the stable DPPH radical method. The advantage of this assay is that the DPPH radical is commercially available and does not need to be generated before the assay as in other assays. The assay was conducted at 1 mM concentration of antioxidants in a polar homogeneous medium. The DPPH radical scavenging capacity of the synthesized capsiate analogues, carried out in the present work, is shown in **Table 2**. Results indicate good radical scavenging activity of the synthesized compounds approaching those of the two reference compounds. DPPH is a well-known radical scavenging assay involving a nitrogen-centered radical, which can react with phenolic OH either through abstracting a hydrogen atom from phenolics or by electron transfer from phenolics and phenoxide anions depending on the polarity of the studied medium. In a polar medium, as has been the case in the present study, the electron transfer mechanism predominates over the H-atom abstraction mechanism (*21*). All of the synthesized capsiates exhibited excellent radical scavenging activity; however, reference antioxidants showed superiority. Very similar activity was observed among the shorter chain homologues (**a**−**d**), and a decreasing trend was observed beyond that (**e**−**i**). Such nonlinear dependency of lipophilicity on antioxidant activity is coherent with other literature reports (*22*, *23*). The radical scavenging activity of the shorter chain homologues (**a**−**d**) is similar to that of α-tocopherol. As the chain length increases, the synthesized compounds showed significant reduction in activity ($p < 0.001$) compared to α-tocopherol. This decrease in radical scavenging activity with the increase in hydrophobicity may be

Table 3. Induction Time of the Soybean Oil Spiked with and without the Reference Antioxidants and the Synthesized Capsiate Analogues

compound[a]	induction time[b] (h)
SBO	8.76 ± 0.34
a	9.83 ± 0.02 ∗
b	9.59 ± 0.76
c	10.44 ± 0.15 ∗
d	10.60 ± 0.26 ∗
e	10.15 ± 0.58 §
f	9.94 ± 0.25 ∗
g	9.70 ± 0.32 §
h	9.84 ± 0.69
i	10.23 ± 0.56 §
j	9.78 ± 0.37 §
BHT	9.56 ± 0.49
α-tocopherol	9.50 ± 1.33

[a] Refer to **Table 1** for **a** −**j**. [b] Values are mean ± SD (n = 3). Synthesized capsiates exhibited significantly increased induction time versus control (soybean oil, SBO) as shown by ∗, $p < 0.01$, and §, $p < 0.02$.

correlated with the decrease in solubility of the analogues in polar medium. Among the synthesized analogues, vanillyl ricinoleate (compound **j**) was found to exhibit the maximum radical scavenging activity, on par with that of BHT and significantly higher than that of α-tocopherol ($p < 0.001$). This may be due to the presence of a hydroxyl group at the hydrophobic chain, influencing their solubility characteristics.

The Rancimat assay is an appropriate in vitro model to evaluate the antioxidant activity of an antioxidant in vegetable oil, and the assay mimics the traditional Indian way of cooking, wherein oil is preheated for 2−3 min at 160−180 °C before the food products to be fried are added. During this time, degradation (oxidation) of oil may happen depending on the degree of unsaturation of fatty acids present in the oil. In this assay the soybean oil (5.0 g), spiked with antioxidant (1.0 mM) was subjected to accelerated oxidation at 110 °C under a purified air flow rate of 20 L/h. The volatile degradation products are trapped in distilled water, influencing its conductivity. The induction time is defined as the time necessary to reach the inflection point of the conductivity curve. An increase in the induction time indicates increased oxidative stability of soybean oil. The results for the oxidative stability of soybean oil with and without antioxidant, measured as induction time, are shown in **Table 3**. All of the synthesized capsiates inhibit the oxidation of soybean oil as is evident from their significantly higher induction period compared to soybean oil. In fact, the activities of the capsiate analogues are better than those of the references. However, the Rancimat assay also does not show any correlation between the structural characteristics of the alkyl chain and their antioxidant activity as all synthesized capsiates showed a nearly constant antioxidant activity. This is because of the accelerating oxidation condition employed in this assay, which is too drastic to find decreasing antioxidant activity with the increase in lipophilicity, validating the rational model of "polar paradox".

Antioxidants are widely employed in cosmetic and food products to inhibit lipid peroxidation, which otherwise renders the product rancid and decreases the shelf life and nutritional quality of the product. Most of such products exist in a formulated complex emulsion system. The efficacy of an antioxidant in such a matrix depends not only on its chemical reactivity as radical scavenger but also on the orientation of its radical scavenging nucleus, interaction with other food components, and environmental conditions (*24*). The third in vitro assay conducted in the present work is the rate of inhibition of autoxidation of linoleic acid by antioxidant in Tween 20-based micellar medium. Initially the autoxidation of linoleic acid is accompanied by a rapid increase of

Table 4. Rate of Inhibition of Autoxidation of Linoleic Acid to Conjugated Diene (Measured by Absorbance at 234 nm) by Capsiate Analogues and Reference Antioxidant in Tween 20-Based Micellar Medium

	absorbance at 234 nm (AU)[a]															
		capsiate analogues[b]												reference		
time (h)	control	**a**[c]	**b**	**c**	**d**	**e**	**f**[c]	**g**[c]	**h**[c]	**i**[c]	**j**[c]	BHT[c]	tocopherol[c]
0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	3.3 ± 0.27	1.50 ± 0.12	1.17 ± 0.05	1.22 ± 0.16	1.18 ± 0.16	1.15 ± 0.17	0.43 ± 0.05	0.54 ± 0.02	0.63 ± 0.08	1.48 ± 0.05	0.87 ± 0.05	0.35 ± 0.02	0.24 ± 0.11
24	6.7 ± 0.61	1.56 ± 0.12	1.21 ± 0.08	1.61 ± 0.04	1.54 ± 0.22	1.37 ± 0.20	0.62 ± 0.02	0.83 ± 0.07	0.82 ± 0.003	1.73 ± 0.08	0.94 ± 0.06	0.41 ± 0.03	0.35 ± 0.06
36	20.3 ± 1.0	1.80 ± 0.13	1.26 ± 0.06	1.84 ± 0.08	1.86 ± 0.15	1.41 ± 0.28	0.73 ± 0.05	0.81 ± 0.06	0.87 ± 0.04	1.90 ± 0.08	0.89 ± 0.09	0.40 ± 0.03	0.42 ± 0.11
48	12.9 ± 0.5	1.84 ± 0.10	1.27 ± 0.04	1.92 ± 0.06	2.33 ± 0.12	1.31 ± 0.41	0.66 ± 0.02	0.82 ± 0.08	0.88 ± 0.05	2.11 ± 0.15	1.10 ± 0.03	0.39 ± 0.04	0.46 ± 0.02
60	12.9 ± 0.6	1.80 ± 0.05	1.36 ± 0.10	2.11 ± 0.05	2.45 ± 0.17	1.60 ± 0.27	1.08 ± 0.04	0.90 ± 0.10	0.98 ± 0.04	2.14 ± 0.06	1.07 ± 0.03	0.88 ± 0.06	0.29 ± 0.07
72	12.5 ± 0.5	1.85 ± 0.06	1.51 ± 0.12	2.33 ± 0.05	2.48 ± 0.10	1.54 ± 0.20	1.39 ± 0.04	0.94 ± 0.09	1.07 ± 0.07	2.15 ± 0.09	1.11 ± 0.04	0.81 ± 0.12	0.46 ± 0.09
84	12.1 ± 0.7	1.91 ± 0.06	1.52 ± 0.06	2.53 ± 0.05	2.55 ± 0.16	1.92 ± 0.23	1.39 ± 0.05	1.07 ± 0.07	1.02 ± 0.04	2.35 ± 0.14	1.16 ± 0.04	0.89 ± 0.12	0.48 ± 0.07
96	12.1 ± 0.6	1.92 ± 0.07	1.51 ± 0.10	2.61 ± 0.06	2.50 ± 0.15	1.86 ± 0.16	1.41 ± 0.04	1.11 ± 0.10	1.00 ± 0.07	2.44 ± 0.15	1.01 ± 0.07	0.93 ± 0.11	0.67 ± 0.04
108	11.5 ± 0.4	2.03 ± 0.03	1.58 ± 0.18	2.53 ± 0.04	2.36 ± 0.16	1.76 ± 0.13	1.30 ± 0.08	1.20 ± 0.10	0.92 ± 0.007	2.54 ± 0.11	1.02 ± 0.11	0.87 ± 0.06	0.86 ± 0.04
120	11.1 ± 0.8	2.04 ± 0.06	1.73 ± 0.17	2.53 ± 0.05	2.50 ± 0.18	2.12 ± 0.12	1.52 ± 0.04	1.19 ± 0.10	0.98 ± 0.02	2.56 ± 0.10	1.09 ± 0.10	0.81 ± 0.10	1.27 ± 0.12

[a] All antioxidants (synthetics and references) exhibited significant inhibition versus control: $p < 0.001$. [b] Refer to **Table 1** for **a**−**j**. Values are mean ± SD (n = 3). [c] Compounds **f**, **g**, **h**, **j**, and references showed increased inhibition versus compound **a**: $p < 0.001$. Compound **i** showed decreased inhibition after 36 h onward versus compound **a**: $p < 0.01$.

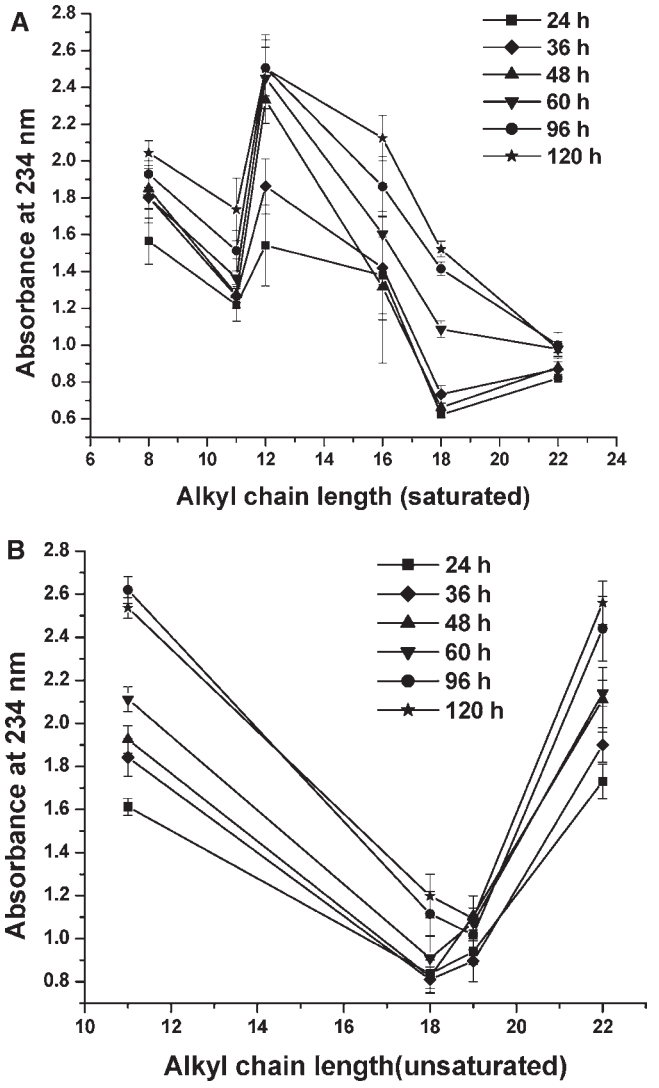


Figure 1. Formation of conjugated diene over 120 h during autoxidation of linoleic acid, measured as absorbance at 234 nm versus the chain length of fatty acids esterified to the vanillyl alcohol: (**A**) saturated analogues; (**B**) unsaturated analogues (vanillyl ricinoleate is represented as C-19).

conjugated diene, which is measured by UV at 234 nm. In the control sample, the formation of conjugated diene was found to reach a maximum in 36 h (**Table 4**). The autoxidation of linoleic acid was found to be markedly inhibited ($p < 0.001$) due to the addition of reference antioxidants as well as synthesized capsiates. This is evident from the negligible formation of conjugated diene in 36 h. When the formation of conjugated diene over 120 h, measured as absorbance at 234 nm, is plotted against the chain length of fatty acids esterified to the vanillyl alcohol, a minimum is obtained near C18 chain length (**Figure 1**). This is observed both in the saturated (**Figure 1A**) and in the monounsaturated series (**Figure 1B**). This indicates that in the studied Tween 20 micellar system, vanillyl ester attached with a C18 alkyl chain (**f**, **g**, and **j**) irrespective of functional moiety exhibited maximum inhibition of autoxidation of linoleic acid, significantly higher ($p < 0.001$) than that of **a** when the study was conducted over a period of 5 days. Further increase in alkyl chain length to C22 reduces the antioxidant capacity significantly (**g** vs **i**) in the unsaturated series (**Figure 1B**). In the saturated series, such a decrease in activity was observed only after 60 h (**f** vs **h**, $p < 0.001$), and even then the activity of **h** is significantly higher than that of **a** ($p < 0.001$). Laguerre et al. (*25*) recently proposed a nonlinear dependency of lipophilicity of chlorogenic acid alkyl ester on antioxidant activity. The study is based on conjugated autoxodizable triene (CAT) assay and observed increased antioxidant activity with increased lipophilicity, reaching a maximum for the dodecyl chain, beyond which the activity decreases with the increase in lipophilicity. Following the same assay, the same authors studied the rosmarinic acid alkyl esters and reported maximum antioxidant efficiency for the corresponding octyl ester homologue (*26*). To support their hypothesis of efficient comicellization as a possible reason for antioxidant efficiency, Lucas et al. (*27*) studied the surface properties of tyrosol and hydroxytyrosol alkyl ester and found maximum surfactant effectiveness in compounds having 8−10 carbon atoms. On the other hand, hydroxytyrosol butyrate showed maximum antioxidant activity in cell-culture DCF assay (*22*), and hydroxytyrosol linoleate showed maximum activity in brain homogenate among their studied homologues (*19*). Thus, the influence of alkyl chain length on antioxidant activity in a micellar or biological system is contrasting in nature. All of these contrasting literature reports indicate that the dependency of antioxidant activity on lipophilicity is related not only to assay type and the structure of the phenolic moiety but also to the physical location and orientation of the radical scavenging nucleus in such a three-phase system. In the case of synthetic capsiates, homologues with lipophilia values similar to that of the natural one (8−10 carbon atoms) are reported to be most active biologically (*10*). However, in the studied micellar system, consisting of nonpolar micellar interior, bulk polar, and the micellar interface, the specific partitioning of vanillyl stearate, oleate, and ricinoleate at the interface is appropriate to counteract the free radical initiated oxidation of linoleic acid compared to other homologues.

In conclusion, lipase-catalyzed synthesis of vanillyl ester of fatty acids was carried out in the present work to synthesize 10 capsiate analogues. All of the analogues exhibited excellent antioxidant activity in the three studied in vitro antioxidant assays. Both DPPH and Rancimat assays did not show any specific trend of antioxidant activity with the increase in lipophilicity or with the type of fatty acid esterified to vanillyl alcohol. In the Tween 20 micellar system for the inhibition of autoxidation of linoleic acid, longer chain homologues (vanillyl stearate, oleate, and ricinoleate) exhibited maximum inhibition of autoxidation of linoleic acid.

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