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Antioxidant Activity of Capsinoids

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Hot peppers are a good source of dietary antioxidants, encompassing, apart from widespread compounds (flavonoids, phenolic acids, carotenoids, vitamin A, ascorbic acid, tocopherols), also specific constituents such as the pungent capsaicinoids (capsaicin, dihydrocapsaicin, and related analogues). We have shown that capsinoids also show remarkable antioxidant activity. These benign analogues of capsaicin could protect linoleic acid against free radical attack in simple in vitro systems, inhibiting both its autoxidation and its iron- or EDTA-mediated oxidation. These properties were retained in some simple synthetic analogues (vanillyl nonanoate and its dimerization products). Capsiate, dihydrocapsiate, and their analogues were devoid of pro-oxidant activity and showed a highly significant antioxidant activity in all systems investigated. Vanillyl nonanoate, a simple capsinoid mimic, was also tested on cell cultures for cytotoxic activity and the capacity to inhibit $FeCl_3$-induced oxidation.

KEYWORDS: Antioxidants; capsinoids; linoleic acid oxidation; capsiate; dihydrocapsiate; vanillyl nonanoate

INTRODUCTION

Antioxidants are widely employed to increase the shelf life of lipids and lipid-containing products, and the discovery of new natural antioxidants has obvious nutritional and pharmaceutical relevance. It is therefore hardly surprising that a large number of plants have been screened as a viable source of these compounds. Peppers (*Capsicum* ss. vv.) are popular as spices and vegetable foods and are a remarkable source of antioxidants (*1*−*3*), including flavonoids (quercetin, luteolin), phenolic acids, carotenoids (*β*-carotene, capxanthin, zeaxanthin), and vitamins (vitamin A, ascorbic acid, tocopherols) (*4*, *5*). The major pungent components in *Capsicum* plants are capsaicin [(*E*)-*N*-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide] and dihydrocapsaicin, the archetypal capsaicinoids (*6*). These compounds also show an interesting antioxidant activity. Thus, capsaicin and dihydrocapsaicin can inhibit iron-mediated lipid peroxidation and copper-dependent oxidation of low-density lipoprotein (*7*), an effect ascribed to their capacity to form complexes with reduced metals and act as hydrogen donors. Capsaicin could also prevent the oxidation of oleic acid at cooking temperatures (*8*) as well as the formation of lipid hydroperoxides from the autoxidation of linoleic acid (*9*). Despite these intriguing findings, the use of capsaicinoids as food antioxidants is obviously limited by their strong pungency and obnoxious properties.

Recently, two capsaicinoid-like substances, capsiate and dihydrocapsiate, have been obtained from the fruits of a nonpungent cultivar of *Capsicum annuum* L. (CH-19 Sweet) (*10*). Capsiate [4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate] and dihydrocapsiate [4-hydroxy-3-methoxybenzyl 8-methylnonanoate] are nonpungent ester analogues of capsaicin and dihydrocapsaicin and are the archetypal capsinoids (*6*). Capsinoids are nonoffensive and devoid of pungency. Nevertheless, they have been shown to share some of the biological activities of capsaicinoids. Thus, it has been reported that nonpungent ester-type analogues of capsaicin enhance adrenal catecholamine secretion (*11*). Furthermore, capsaicin and capsiate can both affect body temperature (*12*), and a recent study suggests that capsiate can promote energy metabolism and suppress body fat accumulation essentially in the same way as capsaicin (*13*). These findings prompted us to investigate the antioxidant activity of capsinoids in several models of lipid oxidation, assaying two natural capsinoids, capsiate (**1**) and dihydrocapsiate (**2**), and three synthetic analogues, vanillyl nonanoate [4-hydroxy-3-methoxybenzyl-nonanoate] (**3**) and its dimers **4** and **5** (DVN 4 and DVN 5) (**Figure 1**). The ability to inhibit lipid peroxidation was investigated in in vitro systems during both the autoxidation and the iron- or EDTA-mediated oxidation of linoleic acid at 37 °C in the absence of solvent. The oxidation pattern was followed by monitoring mainly two parameters: the consumption of the fatty acid and the formation

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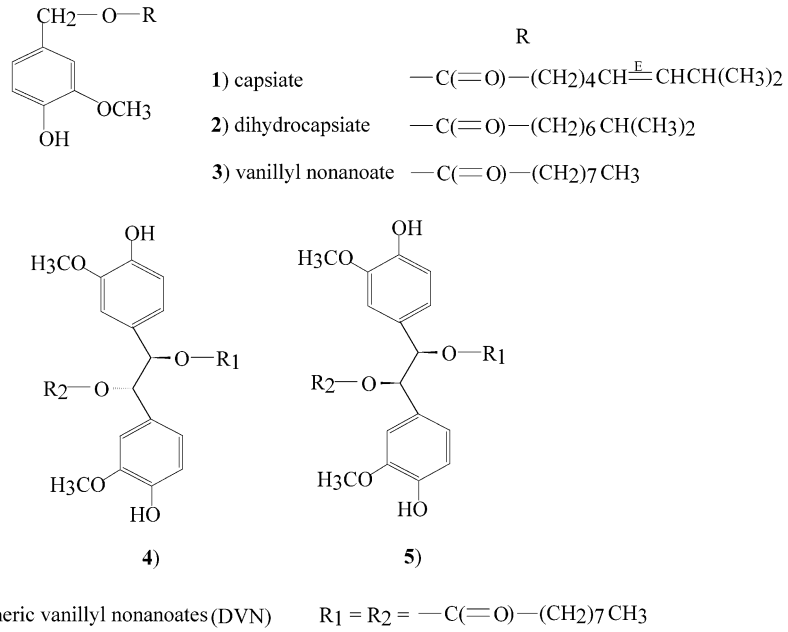

Figure 1. Structures of capsiate (**1**), dihydrocapsiate (**2**), vanillyl nonanoate (**3**), dimeric vanillyl nonanoate (**4**), and dimeric vanillyl nonanoate (**5**).

of its major oxidation products, the hydroperoxyoctadecadienoic acid isomers (HPODEs). The activity of the compounds was compared to that of α-tocopherol, luteolin, and the synthetic antioxidant BHT.

Vanillyl nonanoate, a simplified analogue of natural capsiates, was also assayed for in vitro cytotoxicity and for the capacity to inhibit the oxidative stress induced by $FeCl_3$.

MATERIALS AND METHODS

Chemicals. All solvents used, of the highest available purity, were purchased from Merck (Darmstadt, Germany). Linoleic acid, EDTA (ethylenediaminetetraacetic acid dipotassium salt), 2-thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Milano, Italy). $FeCl_3$ and BHT (butylated hydroxytoluene) were supplied by Carlo Erba (Milano, Italy). *cis*,*trans*-13-Hydroperoxyoctadecadienoic acid (c,t-13-HPODE) and *cis*,*trans*-9-hydroperoxyoctadecadienoic acid (c,t-9-HPODE) were purchased from Cascade (Cascade Biochem. Ltd., London). Luteolin was supplied by Extrasynthese (Genay Cedex, France), and α-tocopherol was purchased from Fluka AG (Buchs, Switzerland). All the cell culture materials were purchased from Life Technologies (Milano, Italy). All the other chemicals used in this study were of analytical grade.

Tested Compounds and Structural Determination. Capsiate and dihydrocapsiate were synthesized from vanillol and their corresponding acids (*10*). Vanillyl nonanoate was synthesized as previously reported (*14*). The two dimeric vanillyl nonanoates (DVN 4 and DVN 5) were obtained by pinacol coupling of *O*-benzylvanillin and esterification (*15*).

Capsiate, dihydrocapsiate, and vanillyl nonanoate were characterized by spectroscopic methods (^{1}H NMR, ^{13}CNMR, UV, IR, and HRMS).

Linoleic Acid Assay. *Oxidation of Linoleic Acid*. Oxidation trials were conducted as previously described by Banni et al. (*16*) with a few modifications. First, 0.5 mL (3566 nmol) of linoleic acid solution (2 mg/mL of MeOH) was dried in a round-bottom test tube under vacuum. The samples were incubated in a water bath at 37 °C for 32 h; controls were kept at 0 °C. Artificial light exposure was kept throughout the experiment. In a different set of experiments, EDTA or $FeCl_3$ was dissolved in solution along with linoleic acid, adjusting the total volume to 1 mL with MeOH, and processed as described above. A 25-μL portion of EDTA solution (1 mg/mL of MeOH) or 54.1 μL of $FeCl_3$ solution (100 μg/mL of MeOH) was added to 0.5 mL of linoleic acid solution (2 mg/mL of MeOH) and incubated at 37 or 0 °C for 32 or 16 h, respectively. The reaction was stopped by cooling and adding 1 mL of CH_3CN/0.14% of CH_3COOH (v/v). Aliquots of the samples were injected into the HPLC system.

Oxidation of Linoleic Acid in the Presence of Capsinoids. Immediately prior to use, in consideration of their instability in polar solvents (*14*), capsiate, dihydrocapsiate, vanillyl nonanoate, DVN 4, and DVN 5 were dissolved in MeOH (1 mg/mL). Different concentrations of the methanol solutions of pure compounds were preincubated with linoleic acid before its autoxidation and oxidation in the presence of $FeCl_3$ or EDTA. BHT, luteolin, and α-tocopherol in methanol solution (1 mg/mL) were tested as reference compounds.

HPLC Analyses. Analyses of linoleic acid and its oxidation products were carried out with a Hewlett-Packard 1050 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a 1040 M diode array detector. An Alltech Adsorbosphere C-18 column (Alltech Europe, Eke, Belgium), 5 μm particle size, 250 × 4.6 mm, was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 mL/min. Linoleic acid was detected at 200 nm; the four HPODE isomers, c,t-9-HPODE, t,t-9-HPODE, c,t-13-HPODE, and t,t-13-HPODE, were detected at 234 nm. Under these chromatographic conditions, one peak is evident corresponding to the mixture of c,t-13-HPODE and c,t-9-HPODE (*16*). The identification of the fatty acid and HPODEs was made using standard compounds and second derivative as well as conventional UV spectra, generated using the Phoenix 3D HP Chemstation software, as detailed in a previous paper (*16*).

Cell Culture Assay. *Cell Culture*. Human embryonic lung fibroblasts (HELF), kindly donated by Prof. V. Vannini (University of Pavia, Italy), were used as a finite cell line. HELF cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin−streptomycin in a humidified atmosphere of 5% CO_2. The medium was changed every third day.

Cytotoxicity Test. The cytotoxic effect of vanillyl nonanoate on HELF cells was assessed by the MTT method (*17*, *18*). Prior to confluence, cells were transferred at about 20 × 10^3 cells/well to 24-well plates in 1 mL of DMEM and cultured overnight. Vanillyl nonanoate was dissolved immediately before use to a concentration of 1% in ethanol and then added to the complete medium. Increasing concentrations (5, 10, 50, 100, or 250 μM) of the solution of vanillyl nonanoate, or an equivalent volume of ethanol for the controls, were added to cells and incubated for further 24 h. An 80-μL portion of MTT solution (5 mg/mL of PBS) was then added and left for 4 h at 37 °C. The medium was aspirated, and 1 mL of DMSO was added to the wells. Color development was measured in the supernatant at 570 nm with a Hewlett-Packard 8254A spectrophotometer.

TBARS Test. Oxidation stress was induced by $FeCl_3$. Prior to confluence, cells were transferred at about 50 × 10^4 cells/flask. Twenty-four hours later, the medium was changed to PBS, and two concentra-

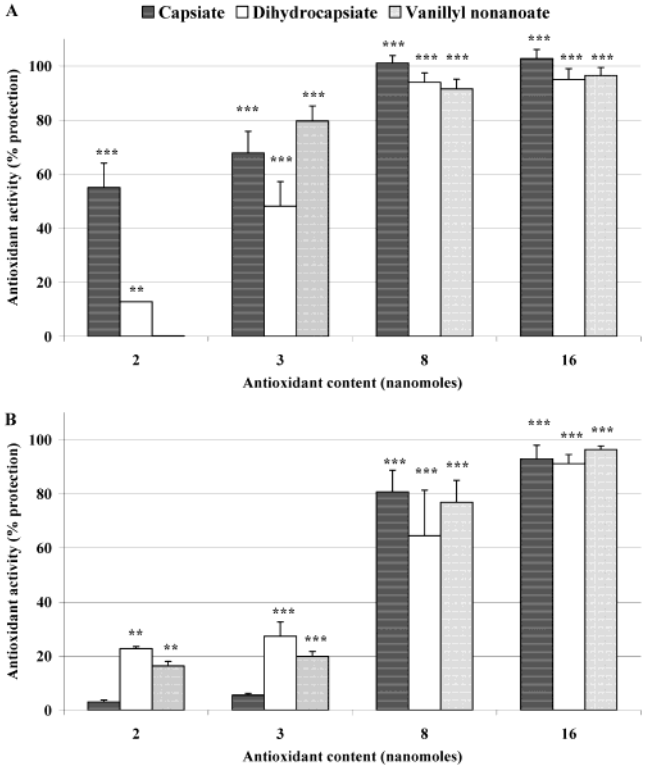

Figure 2. (A) Antioxidant activity, expressed as % protection, of different amounts of capsiate, dihydrocapsiate, and vanillyl nonanoate, measured during the autoxidation of linoleic acid at 37 °C for 32 h. (B) Antioxidant activity, expressed as % protection, of different amounts of capsiate, dihydrocapsiate, and vanillyl nonanoate, measured during the oxidation of linoleic acid at 37 °C for 16 h in the presence of $FeCl_3$. ***, $p < 0.001$; **, $p < 0.01$ versus controls ($n = 6$).

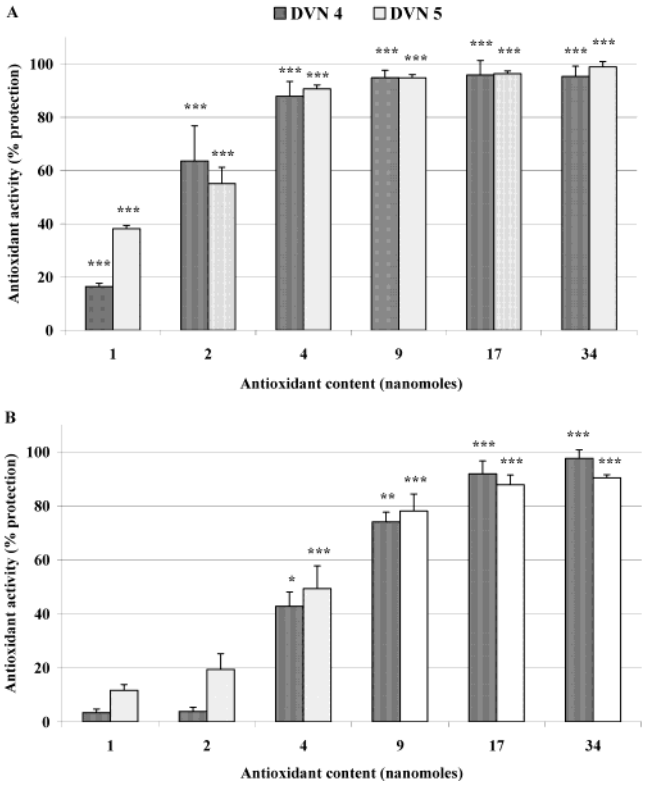

Figure 3. (A) Antioxidant activity, expressed as % protection, of different amounts of dimeric vanillyl nonanoate (**4**) (DVN 4) and dimeric vanillyl nonanoate (**5**) (DVN 5), measured during the autoxidation of linoleic acid at 37 °C for 32 h. (B) Antioxidant activity, expressed as % protection, of different amounts of DVN 4 and DVN 5, measured during the oxidation of linoleic acid at 37 °C for 16 h in the presence of $FeCl_3$. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ versus controls ($n = 6$).

tions of vanillyl nonanoate (1.5 or 2.5 μM), or an equivalent volume of ethanol for the controls, were added. After 2 h of incubation, different concentrations of $FeCl_3$ (200 and 400 μM) aqueous solutions were added and left for 1 h more. The extent of oxidation was evaluated as MDA formation, measured with the TBARS method (*19*).

Statistical Analyses. Data are presented as means ± standard deviation of triplicate values obtained in two or three independent experiments ($n = 6$ or 9). Statistical significance within sets of data was determined by one-way analysis of variance with the ANOVA program. It uses the Bonferroni method: the threshold for significance is the traditional value ($p < 0.05$) divided by the number of comparison.

RESULTS

Antioxidant Activity on Linoleic Acid Assay. Natural capsinoids, capsiate (**1**) and dihydrocapsiate (**2**), and their synthetic analogues (**3**−**5**, **Figure 1**) were assayed for antioxidant activity. A series of in vitro systems developed to evaluate the antioxidant properties of natural extracts were employed (*20*). Luteolin and α-tocopherol, both contained in *C. annuum* (*1*, *5*), and the synthetic antioxidant BHT were employed as references.

Results obtained with the autoxidation and the iron-catalyzed oxidation of linoleic acid in the presence of different amounts of capsiate, dihydrocapsiate, and vanillyl nonanoate are reported in **Figure 2**. Antioxidant activity was expressed as percentage of protection of linoleic acid, calculated according to the difference in the level of fatty acid in protected and unprotected systems. All compounds were active in these systems. Complete inhibition of the oxidation was observed at 8 nmol, corresponding to a molar ratio of compound to fatty acid of 1:446, during the autoxidation of linoleic acid (**Figure 2A**) and 16 nmol (1:223) during the oxidation in the presence of iron (**Figure 2B**). Data obtained during the autoxidation and the iron-mediated oxidation of linoleic acid in the presence of different amounts of DVN 4 and DVN 5 are reported in **Figure 3**. These compounds gave a total inhibition of the oxidation process from 8 nmol (1:446) during the autoxidation of linoleic acid (**Figure 3A**) and 17 nmol (1:209) during the oxidation in the presence of iron (**Figure 3B**). The addition of a small amount of EDTA to the system enhanced the antioxidant activity (data not shown), presumably as a result of the chelation of metal ions. In contrast to α-tocopherol (*16*), none of the compounds showed any prooxidant activity at high concentrations.

The IC_{50} values of the compounds tested are presented in **Table 1**. Data observed with α-tocopherol, luteolin, and the synthetic antioxidant BHT are reported for comparison. In the autoxidation of linoleic acid, BHT was the most powerful inhibitor on a molar basis, with an IC_{50} of 0.7 nmol. Capsiate and dimeric vanillyl nonanoates, though less effective, still exerted a significant antioxidant activity, comparable to that of luteolin and α-tocopherol. The activity in preventing the oxidation process of dihydrocapsiate and vanillyl nonanoate is also remarkable (IC_{50} of 3.0 and 2.5 nmol, respectively).

Also in the test of the iron-catalyzed oxidation of linoleic acid, BHT was the most powerful compound (IC_{50} of 1.9 nmol). Apart from α-tocopherol, all the other compounds showed a great efficiency in protecting linoleic acid against free radical attack, the order being DVN 5 and DVN 4 (IC_{50} of 4.3 and 4.9 nmol, respectively) > capsiate, vanillyl nonanoate > dihydrocapsiate > luteolin (IC_{50} of 12 nmol).

Table 1. IC_{50} Values (Amount That Gives a Protection of 50%) Calculated during Linoleic Acid Autoxidation and Iron-Mediated Oxidation

compound	IC_{50} (nmol) linoleic acid autoxidation	IC_{50} (nmol) linoleic acid oxidation in the presence of $FeCl_3$
capsiate	<1.63	5.7
dihydrocapsiate	3.0	6.3
vanillyl nonanoate	2.5	5.7
dimeric vanillyl nonanoate 4	1.3	4.9
dimeric vanillyl nonanoate 5	1.4	4.3
luteolin	1.4	12.0
BHT	0.7	1.9
α-tocopherol	1.7	27.5

Table 2. Values of the Ratio of Hydroperoxyoctadecadienoic Acids (HPODE) c,t to t,t Isomers Formed during Linoleic Acid Autoxidation and Oxidation Mediated by $FeCl_3$, in the Presence of Capsiates (**1**), Dihydrocapsiate (**2**), Vanillyl Nonanoate (**3**), Dimeric Vanillyl Nonanoates (**4** and **5**), Luteolin, BHT, and α-Tocopherol[a]

Reference Values: HPODE c,t/HPODE t,t	
18:2	3.59
18:2 ox	1.35
18:2 ox + $FeCl_3$	1.24

HPODE c,t/HPODE t,t

amount of compd (μg)	1	2	3	4	5	BHT	luteolin	α-tocopherol
Linoleic Acid Autoxidation								
0.5	1.30	1.66	1.68	1.35	1.22	3.28	1.26	1.24
1	1.26	1.50	1.55	1.28	1.22	3.09	2.28	1.20
2.5	1.61	1.58	1.45	1.40	1.48	3.78	2.10	2.25
5	2.57	1.75	1.66	1.66	1.57	3.89	2.20	6.35
10	3.45	2.49	1.94	1.88	1.70	4.79	2.26	6.41
20	4.55	3.03	3.08	2.68	2.87	4.75	2.51	12.91
30	5.07	3.58	3.10	3.67	3.75	5.05	2.64	11.55
50	4.98	4.90	3.80	2.47	5.47	4.52	3.07	13.23
100	7.31	5.59	6.17	2.46	6.39	5.44	3.71	20.28
Linoleic Acid $FeCl_3$-Mediated Oxidation								
0.5	1.20	1.29	1.34	1.14	1.24	1.20	1.26	nd
1	1.26	1.27	1.32	1.27	1.25	2.41	1.23	1.21
2.5	1.33	1.27	1.34	1.27	1.30	3.63	1.25	1.24
5	1.52	1.49	1.63	1.38	1.38	4.85	1.23	1.24
10	1.66	1.90	2.12	1.49	1.73	4.31	1.58	1.22
20	3.10	2.64	2.50	1.91	2.22	5.41	1.82	1.21
30	4.72	3.61	2.96	2.23	2.82	23.42	2.75	1.21
50	5.06	3.55	3.89	3.11	3.91	17.58	2.58	1.19
100	5.89	5.82	5.01	2.22	6.98	16.33	5.00	5.22

[a] Reference values obtained for initial linoleic acid (18:2), during linoleic acid autoxidation (18:2 ox), and during oxidation in the presence of $FeCl_3$ (18:2 ox + $FeCl_3$) are also reported.

The values for the HPODEs isomers, c,t-9-HPODE, t,t-9-HPODE, c,t-13-HPODE, and t,t-13-HPODE, were measured in all experimental systems. The two c,t isomers were added as well as the t,t isomers, and the ratio (c,t/t,t) was calculated for all the tested compounds. Data obtained during linoleic acid autoxidation and oxidation in the presence of $FeCl_3$ are reported in **Table 2**. During linoleic acid autoxidation, only α-tocopherol gave a slight shift of the c,t/t,t ratio at the highest concentrations, while capsiate, dihydrocapsiate, vanillyl nonanoate, DVN 5, and BHT gave a clear shift versus the c,t isomers at increasing concentrations. During linoleic acid oxidation in the presence of $FeCl_3$, BHT gave a slight shift of the c,t/t,t ratio at the highest concentrations, but capsiate, dihydrocapsiate, vanillyl nonanoate, DVN 5, and luteolin gave a clear shift versus the c,t isomers at increasing concentrations.

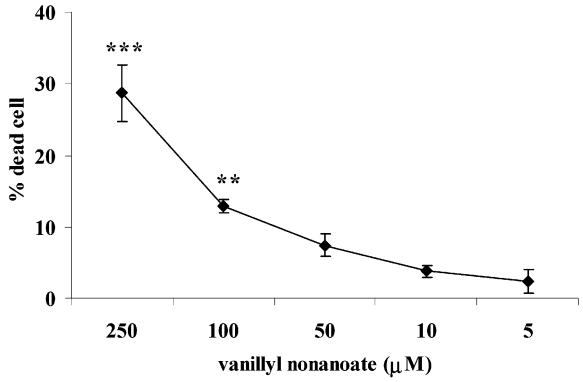

Figure 4. Percentage of dead cells induced after 24 h of incubation with different concentrations of vanillyl nonanoate. ***, $p < 0.001$; **, $p < 0.01$ versus controls ($n = 9$).

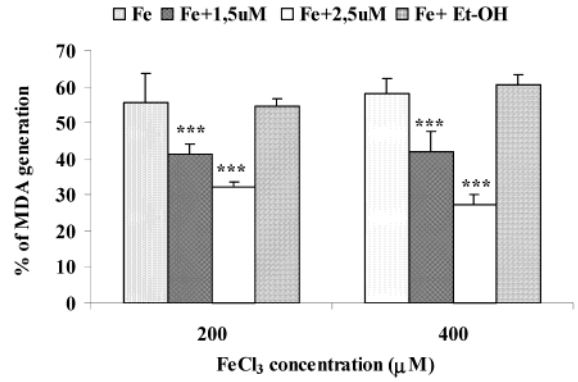

Figure 5. Percentage of MDA formation induced by $FeCl_3$ in the presence of vanillyl nonanoate (1.5 and 2.5 μM in 1% ethanol solution). ***, $p < 0.001$ versus controls ($n = 9$).

Cytotoxic Activity. Vanillyl nonanoate was tested in cell culture to evaluate its cytotoxic activity. A set of experiments was performed to determine the nontoxic concentration. **Figure 4** shows the percentage of dead cells after 24 h of incubation in the presence of vanillyl nonanoate compared to the controls. Vanillyl nonanoate exerted a significant cytotoxic activity only at concentrations higher than 100 μM. At the concentrations employed for the antioxidant assays (1.5 and 2.5 μM), the percentage of dead cells was below 2%.

Antioxidant Activity on Cell Culture. We have determined the antioxidant activity of vanillyl nonanoate on oxidative damage induced on HELF cells by $FeCl_3$. Oxidation increased in a dose-dependent manner with increasing concentrations of $FeCl_3$. Concentrations of 200 and 400 μM for $FeCl_3$ were selected, since these concentrations yielded highly significant levels of lipid peroxidation. The extent of oxidative damage was measured as MDA production. The antioxidant activity of vanillyl nonanoate was evaluated in the same experimental system and expressed as inhibition of MDA formation compared to the controls. **Figure 5** shows that vanillyl nonanoate (1.5 or 2.5 μM) inhibited $FeCl_3$-induced lipid peroxidation in a dose-dependent manner. Both concentrations of vanillyl nonanoate were active against oxidative damage and showed a significant protection against the MDA generation. The antioxidant effect of vanillyl nonanoate is not related to the presence of ethanol.

DISCUSSION

Several capsinoids were assayed for their capacity to protect linoleic acid against the attack of peroxyl radicals, during both the autoxidation and the oxidation in the presence of iron or EDTA. In these systems, the major oxidation products of linoleic acid are its HPODEs isomers and the peroxyl radicals formed by their degradation, an event promoted by Fe^{3+} ions. The oxidation pattern was followed by monitoring mainly two parameters: the consumption of the fatty acid and the formation of its HPODEs. The activity of capsinoids was compared with that of α-tocopherol, luteolin, and BHT.

All tested compounds were active in these systems, and none of them showed any pro-oxidant activity. Significant differences in the efficacy of the tested compounds as antioxidants were observed, with BHT showing the major antioxidant activity in all systems. During linoleic acid autoxidation, capsiate and the two dimeric vanillyl nonanoates exerted a highly significant antioxidant activity, comparable to that of luteolin and α-tocopherol. Under the same experimental conditions, dihydrocapsiate and vanillyl nonanoate were less effective but still showed an interesting preventive activity in the autoxidation process.

During the iron-catalyzed oxidation of linoleic acid, where an additional chelating activity was observed, the two dimeric vanillyl nonanoates, capsiate, dihydrocapsiate, and vanillyl nonanoate showed a comparable and notable activity in protecting linoleic acid against free radical attack. In this system, luteolin and α-tocopherol were active only at high concentrations.

Using these in vitro systems, it is possible to discriminate geometrical HPODEs isomers formed during linoleic acid oxidation and, by focusing on the ratio of the HPODEs formed (c,t/t,t), to point out any hydrogen-atom-donating activity (*16*). The formation of HPODE isomers during the oxidation process shows a paraboloid pattern, with an initial shift versus the c,t isomers that disappears with the fatty acid degradation, but it is enhanced by the presence of a strong hydrogen atom donator (*16*). A slight and concentration-dependent shift of the c,t/t,t ratio was evident for α-tocopherol during the autoxidation of linoleic acid and for BHT during the iron-mediated oxidation of the fatty acid. This confirms that the main mechanism of action of these compounds involves a hydrogen-atom-donating activity (*9*, *16*). Capsiate, dihydrocapsiate, vanillyl nonanoate, and DVN 5 showed a similar shift during both the autoxidation and the $FeCl_3$-mediated oxidation, a finding suggesting that their antioxidant activity is partially due to donation of hydrogen's activity from the phenolic moieties. The strong antioxidant activity exerted by these compounds during the iron-mediated oxidation of linoleic acid indicates a more efficient protective effect in the presence of iron compared to luteolin (*21*). This ability to protect against iron-induced oxidative stress was also clearly shown by vanillyl nonanoate, a syntetic chemical model of capsinoids, also on cell cultures. MDA generation was significantly inhibited at concentrations of 1.5 and 2.5 μM.

All capsinoids and their analogues showed, at noncytotoxic concentrations for vanillyl nonanoate, a noteworthy efficacy as chain-braking antioxidants in scavenging lipid peroxyl radicals, an effect due to their capacity to donate hydrogen atoms and delocalize the resulting radical sites.

Taken together, our data show that capsiate and dihydrocapsiate exert antioxidant activity comparable to that of their corresponding capsaicinoids (capsaicin and dihydrocapsaicin). Since capsiate and dihydrocapsiate are devoid of pungency and the other obnoxious properties of capsaicinoids, they fully qualify as interesting antioxidant leads.

Considering the popular use of peppers, these compounds, present in a fairly good amount in the variety CH-19 Sweet of *Capsicum annuum* L., about 98 mg and 59 mg/kg of fresh fruit respectively (*10*), have an evident nutritional relevance.

ABBREVIATIONS USED

DVN, dimeric vanillyl nonanoate; HPODE, hydroperoxyoctadecadienoic acid.

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