Chemical Composition and Antioxidant Activity of Extracts from *Daphne gnidium* L.

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ABSTRACT: Members of the *Daphne* genus have been of interest owing to their excellent medicinal value. In this work, we describe the results of phytochemical analysis and the antioxidant activity of the methanol extracts from leaves and stems of *D. gnidium* L. grown wild in Sardinia, Italy. Four coumarins (daphnetin, daphnin, acetylumbelliferon, and daphnoretin), nine flavonoids (apigenin, luteolin, quercetin, orientin, isoorientin, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, genkwanin, and 5-*O*-β-D-primeverosyl genkwanine), and α-tocopherol were isolated. We investigated the ability of the two extracts and five pure compounds (daphnetin, daphnoretin, apigenin, luteolin, and α-tocopherol) to protect linoleic acid against free radical attack in simple *in vitro* systems by autoxidation and iron- or EDTA-mediated oxidation. Pure compounds were the most active antioxidants. During autoxidation, daphnetin, luteolin, and α-tocopherol were effective at a molar ratio of 1:1600, 1:2500, and 1:2000, respectively. Daphnoretin was active only at high concentrations. During the iron-catalyzed oxidation of linoleic acid, all the materials tested showed activity in the following order: luteolin > daphnetin > α-tocopherol > leaf extract > stem extract > daphnoretin. Apigenin was not active in any of the experimental systems used.

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KEY WORDS: Antioxidants, *Daphne gnidium* L., linoleic acid oxidation, plant extracts.

The addition of antioxidants has become popular as a means of increasing the shelf life of lipids and lipid-containing products. Synthetic antioxidants, such as BHA, BHT, and TBHQ are used in the food and cosmetic industries to improve the stability of their products (1). They are highly effective but may have undesirable effects. As an example, BHA and BHT present in the diet are suspected to be carcinogenic (2–4). For these reasons, the use of natural antioxidants is being widely advocated. A large number of plants have been targeted as sources of natural antioxidants.

We initiated a chemical and biological study on *Daphne gnidium* L. (Thymeleaceae), a plant that commonly grows wild in Sardinia, Italy. Members of the *Daphne* genus have been of great interest owing to their excellent medicinal value. Different classes of natural products have been isolated from these species, including flavonoids, coumarins, and diterpenoids. In folk medicine, some species are widely used for various preparations, and some compounds isolated from these plants show antileukemic activity against the P-388 lymphocytic leukemia in mice (5). *Daphne gnidium* is an evergreen shrub that grows in the Mediterranean area and can grow to a height of 2 m. The methanol extract obtained from the leaves of *D. gnidium* is known to possess antibacterial and antimycotic activities (6,7) and a good cutaneous tolerability, whereas the bark extract induces photochemical skin damage (8). In this paper we report the phytochemical analysis and the antioxidant activity of the methanol extracts from *D. gnidium* leaves and stems and their major constituents. The ability to inhibit lipid peroxidation was investigated in *in vitro* systems for autoxidation and for iron or EDTA-mediated oxidation of linoleic acid at 37°C in the absence of solvent. The oxidation pattern was evaluated with respect to the consumption of the FA and the formation of its hydroperoxyoctadecadienoic acid isomers (HPODE).

EXPERIMENTAL PROCEDURES

Plant material. Daphne gnidium was collected in Burcei, Sardinia, during full blossom in accordance with *Flora d'Italia* (9). A voucher specimen was deposited in the Herbarium of the Department of Botany and Botanical Gardens, University of Cagliari.

Extraction and fractionation procedure. (i) Stems. The stems (300 g, fresh wt) were air dried at 40°C and then powdered and exhaustively extracted by maceration with methanol (MeOH) at room temperature. After evaporation under vacuum, a residue (9.5 g, dry wt) was obtained. The residue, dissolved in MeOH, was brought to pH 8 and extracted with diethyl ether to give a neutral fraction (1.5 g, dry wt). The aqueous solution was acidified with 10% HCl to yield 8 g of crude extract. The crude extract was fractionated on silica gel with hexane/ethyl acetate (EtOAc) and Me_2CO/MeOH mixtures of increasing polarity as eluents. The fraction eluted with EtOAc/hexane (30:70, vol/vol) gave daphnetin (90 mg) and acetylumbelliferon (15 mg). The fraction eluted with EtOAc/hexane (80:20, vol/vol) gave daphnoretin (30 mg). Elution with MeOH/Me_2CO (5:95, vol/vol) followed by gel filtration over Sephadex LH-20 (Fluka, Milano, Italy) in MeOH gave luteolin (50 mg) and genkwanin (27 mg). The MeOH/Me_2CO (40:60, vol/vol) eluates were

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rechromatographed on Sephadex LH-20 with MeOH followed by silica gel with dichloromethane (CH_2Cl_2)/MeOH (70:30, vol/vol) to give orientin (30 mg), isoorientin (38 mg), apigenin 7-*O*-glucoside (41 mg), and daphnin (60 mg). The fraction eluted with MeOH/Me_2CO (90:10, vol/vol) was further subjected to Sephadex LH-20 (H_2O/MeOH, 10:90, vol/vol) column chromatography and gave 5-*O*-β-D-primeverosyl genkwanine (25 mg).

(ii) Leaves. The leaves (200 g, fresh wt) were air dried at 40°C and the dried material was exhaustively extracted with MeOH in a Soxhlet apparatus. After evaporation under vacuum, a residue was obtained. The residue was dissolved in CH_2Cl_2 and filtered. The CH_2Cl_2 extract (10 g) was fractionated on silica gel with petroleum ether/EtOAc mixtures of increasing polarity as eluent. The fraction eluted with petroleum ether gave α-tocopherol (20 mg). The fraction eluted with EtOAc/petroleum ether (30:70, vol/vol) gave daphnetin (180 mg) and acetylumbelliferon (8 mg). The EtOAc/petroleum ether (50:50, vol/vol) eluates gave daphnoretin (15 mg). The residue insoluble in CH_2Cl_2 was dissolved in MeOH. The MeOH extract (15 g) was chromatographed over silica gel with CH_2Cl_2 containing increasing amounts of MeOH. The fraction eluted with MeOH/CH_2Cl_2 (5:95, vol/vol) was purified by further chromatography on silica gel (CH_2Cl_2/MeOH, 20:80, vol/vol) to give luteolin (13 mg). The fraction eluted with MeOH/CH_2Cl_2 (15:85, vol/vol) showed two major spots on TLC that were separated by preparative TLC (HOAc/CH_2Cl_2, 10:1, vol/vol) to give apigenin (0.6 mg) and quercetin (0.5 mg). The fraction eluted with MeOH/CH_2Cl_2 (30:70, vol/vol) was further purified by Sephadex LH-20 gel filtration (MeOH) to give daphnin (10 mg). The MeOH/CH_2Cl_2 (10:90, vol/vol) eluates were rechromatographed on Sephadex LH-20 with MeOH to give luteolin 7-*O*-glucoside (60 mg).

Identification of compounds. Compounds were characterized by spectroscopic methods (UV, FTIR, EI–MS, ^{1}H NMR, and ^{13}C NMR), and their structures were determined by comparison of their physical properties with the values reported earlier for daphnetin, daphnin, daphnoretin (10), acetylumbelliferon (11), apigenin, luteolin, quercetin, orientin, isoorientin, lutein 7-*O*-glucoside, apigenin 7-*O*-glucoside, genkwanin (10,12), 5-*O*-β-D-primeverosyl genkwanine (13), and by direct comparison with the authentic samples preserved in our laboratory.

UV spectra were recorded on a Cintra 5 UV spectrophotometer (GBC Scientific Equipment, Victoria, Australia). FTIR spectra were recorded on a PerkinElmer System 2000 FTIR (PerkinElmer, Buckinghamshire, United Kingdom) using KBr mulls. Mass spectra were taken by a QMD 1000 instrument (Carlo Erba Instruments, Milano, Italy) at 70 eV using a direct inlet system. ^{13}C and ^{1}H NMR spectra of solutions in $CDCl_3$ or DMSO-D_6 of the isolated compounds were also recorded on a Varian VXR-300 spectrometer (Varian, Palo Alto, CA) equipped with a 5-mm NMR tube using tetramethylxylane as an internal standard.

The following types of silica gel were used: 60 H, 400–440 mesh, for column chromatography and 60 PF254 for preparative TLC (Merck, Darmstadt, Germany). Sephadex LH 20 was also used for column chromatography.

Oxidation of linoleic acid. All solvents used were of the highest available purity (Merck). *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, United Kingdom). Oxidation trials were conducted as previously described in Dessì *et al.* (14) with a few modifications. Briefly, 0.5 mL of linoleic acid (Sigma Chemical, St. Louis, MO) solution (2 mg/mL MeOH) was dried down in a round-bottomed 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 maintained throughout the experiment. In a different set of experiments, EDTA (Sigma Chemical) or $FeCl_3$ (Carlo Erba) was dissolved in solution with linoleic acid. The total volume was adjusted to 1 mL with MeOH and processed as described above. A 25 µL quantity of EDTA solution (1 mg/mL MeOH) or 54.1 µL of $FeCl_3$ solution (100 µg/mL MeOH) was added to 0.5 mL of the linoleic acid solution (2 mg/mL 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% CH_3COOH (vol/vol). Aliquots of the samples were injected into the HPLC system.

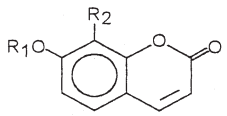
Oxidation of linoleic acid in the presence of plant extracts. Dried methanol extracts, daphnetin, luteolin, apigenin (Extrasynthese, Genay Cedex, France) and α-tocopherol (Fluka) were dissolved in MeOH (1 mg/mL). Different concentrations of the methanol solutions of each extract and pure compounds were preincubated with linoleic acid before their autoxidation and oxidation in the presence of $FeCl_3$ or EDTA. BHT (Carlo Erba) in methanol solution (1 mg/mL) was tested as the reference compound.

HPLC analyses. Analyses of linoleic acid and its oxidation products were carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a 1040M diode array detector (Hewlett-Packard, Palo Alto, CA). A C18 Alltech Adsorbosphere 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, by vol) at a flow rate of 1.5 mL/min. Linoleic acid was detected at 200 nm and HPODE were detected at 234 nm. Spectra (195 to 315 nm) of the eluate were obtained every 1.28 s. Second-derivative spectra, generated using Phoenix 3D HP Chemstation software, as well as conventional UV spectra were taken to confirm the identification of the peaks.

Statistical analyses. Data are presented as mean ± SD of triplicate values obtained in three independent experiments (n = 9). INSTAT software (GraphPad Software, San Diego, CA) was used to determine statistical significance within sets of data by one-way ANOVA.

RESULTS AND DISCUSSION

A preliminary analytical investigation of the two methanol extracts obtained from the stems and the leaves of *D. gnidium*



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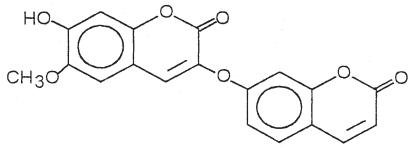

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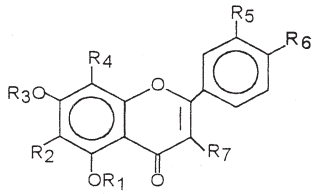

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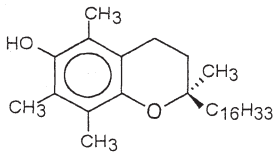

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	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1 daphnetin	H	OH					
2 daphnin	glucosyl	OH					
3 acetylumbelliferon	$COCH_3$	H					
4 daphnoretin							
5 apigenin	H	H	H	H	H	OH	H
6 luteolin	H	H	H	H	OH	OH	H
7 quercetin	H	H	H	H	OH	OH	OH
8 orientin	H	H	H	glucosyl	OH	OH	H
9 isoorientin	H	glucosyl	H	H	OH	OH	H
10 luteolin 7-*O*-glucoside	H	H	glucosyl	H	OH	OH	H
11 apigenin 7-*O*-glucoside	H	H	glucosyl	H	H	OH	H
12 genkwanin	H	H	CH_3	H	H	OH	H
13 5-*O*-β-D-primeverosyl genkwanine	xyloglucosyl	H	CH_3	H	H	OH	H
14 α-tocopherol							

FIG. 1. Structure of major compounds found in the extracts from *Daphne gnidium*.

was performed to identify the major constituents. The stems furnished the known coumarins and flavonoids daphnetin, daphnin, acetylumbelliferon, daphnoretin, apigenin, luteolin, quercetin, orientin, isoorientin, apigenin 7-*O*-glucoside, genkwanin, and 5-*O*-β-D-primeverosyl genkwanine, whereas the leaves gave daphnetin, daphnin, acetylumbelliferon, daphnoretin, luteolin, luteolin 7-*O*-glucoside, and α-tocopherol. Structures of the compounds are reported in Figure 1.

The ability of the two extracts to protect linoleic acid against peroxy radicals attack was tested during both autoxidation and oxidation in the presence of iron or EDTA of linoleic acid at 37°C in the absence of solvent. The major oxidation products of linoleic acid in these systems were HPODE isomers and peroxy radicals formed mainly by Fe^{3+} ion-mediated degradation. The oxidation pattern was followed by monitoring the consumption of the FA and the formation of HPODE. Both extracts were active in these systems. Data obtained during linoleic acid autoxidation are reported in Figure 2. The most active was the leaf extract, which was effective at 2.5 μg (1:400, w/w; extract: FA). About 70% of the initial amount of linoleic acid remained intact after treatment. Full inhibition of the oxidation process was observed in the 5 μg leaf extract treatment. The stem extract was effective at 10 μg (1:100, w/w) and fully inhibited the oxidation process at 20 μg. The addition of EDTA to the system enhanced the antioxidant activity of the materials tested (data not shown). During iron-catalyzed oxidation of linoleic acid, both extracts showed some iron ion-chelating abilities at high concentrations (Fig. 3). However, the leaf extract was again more active than the stem extract. Under these experimental conditions, none of the extracts showed any prooxidant activity, even at a weight ratio of 1:10.

The effect of daphnetin, daphnoretin, apigenin, luteolin, and α-tocopherol, purified from extracts, on linoleic acid oxidation was determined under similar experimental conditions, and their activity was compared with that of BHT. Data obtained during the autoxidation of linoleic acid are reported in Figure 4. With the exception of daphnoretin, pure compounds appeared to protect linoleic acid against free radical attack better than the crude extracts. BHT was the most powerful antioxidant, showing a full inhibition of the autoxidation process at a concentration of 0.5 μg (2.3 nmol). Among the

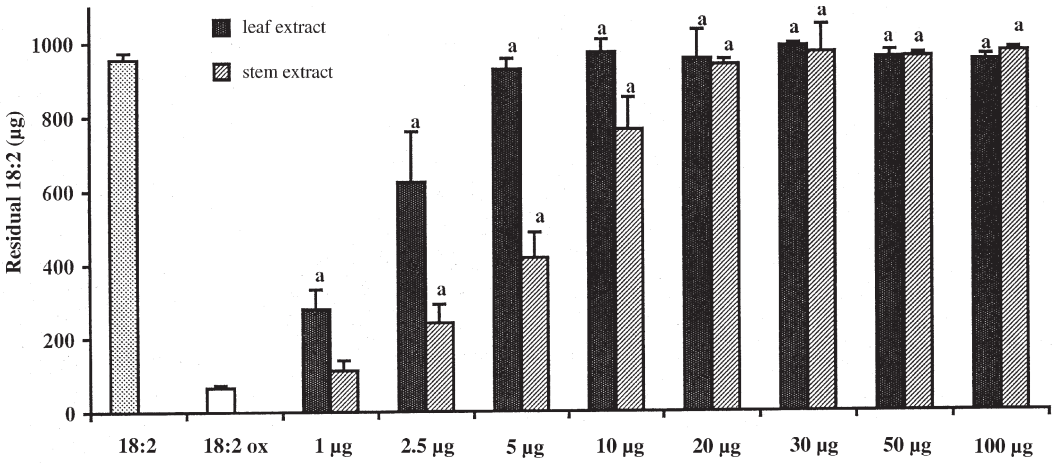

FIG. 2. Autoxidation pattern of linoleic acid at 37°C for 32 h in the presence of various concentrations of the leaf and stem extracts. a = $P < 0.001$ vs. 18:2 ox (residual linoleic acid).

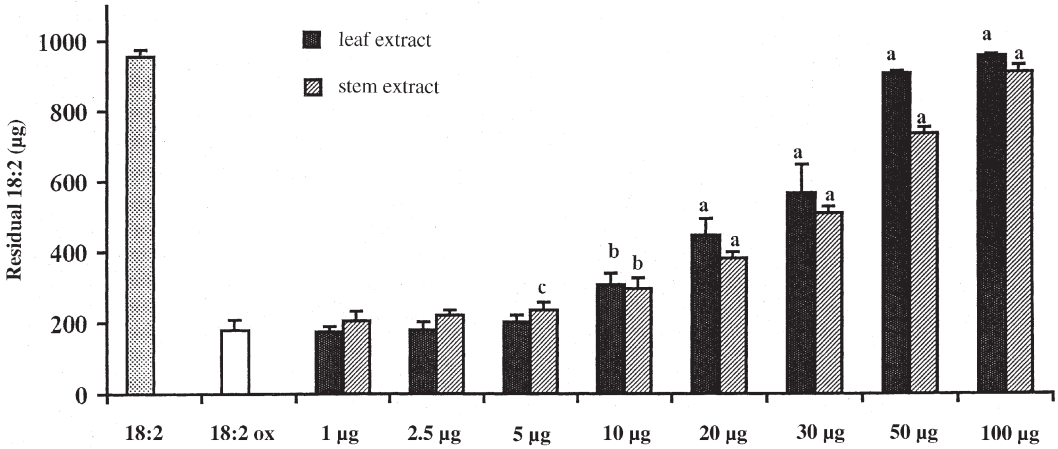

FIG. 3. Oxidation pattern of linoleic acid at 37°C for 16 h in the presence of $FeCl_3$ and various concentrations of the leaf and stem extracts. a = $P < 0.001$; b = $P < 0.01$; c = $P < 0.05$ vs. 18:2 ox (residual linoleic acid).

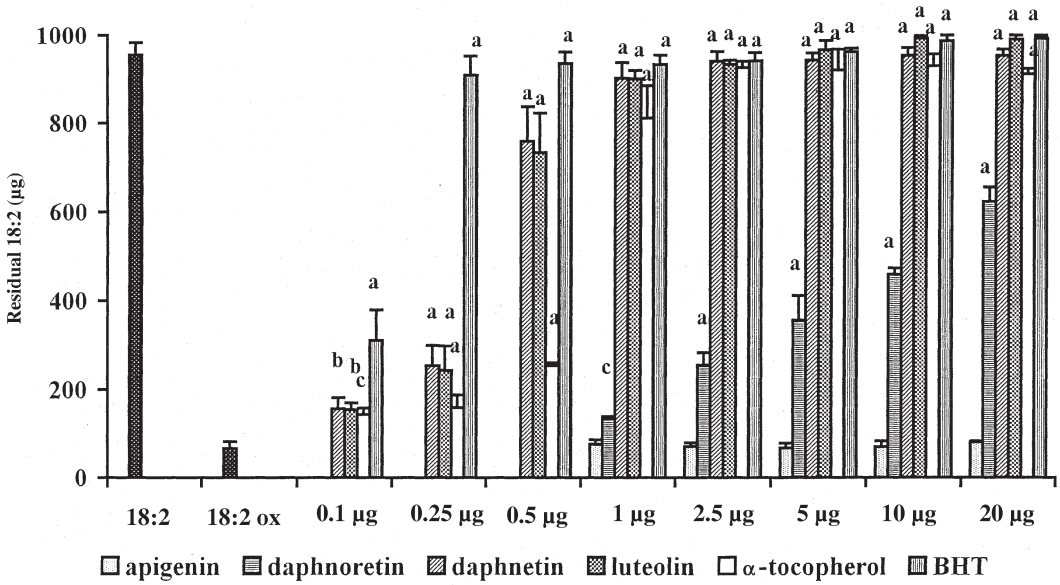

FIG. 4. Autoxidation pattern of linoleic acid at 37°C for 32 h in the presence of various concentrations of daphnetin, daphnoretin, apigenin, luteolin, α-tocopherol, and BHT. a = $P < 0.001$; b = $P < 0.01$; c = $P < 0.05$ vs. 18:2 ox (residual linoleic acid).

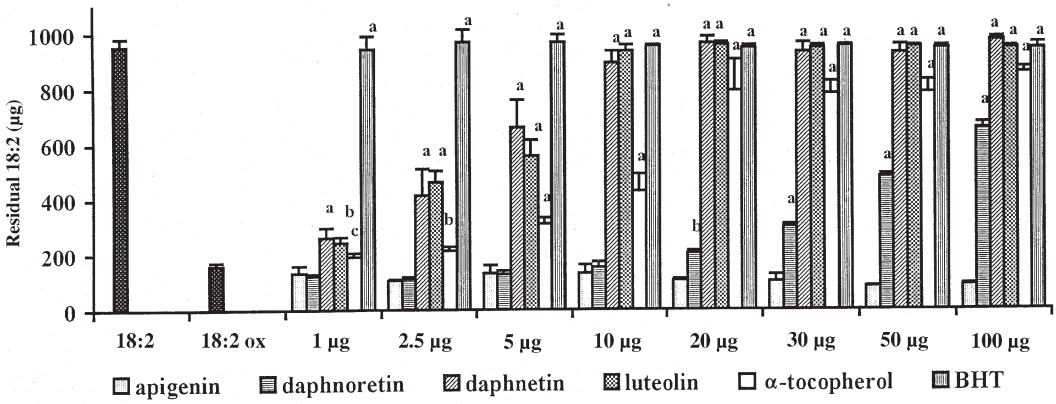

FIG. 5. Oxidation pattern of linoleic acid at 37°C for 16 h in the presence of $FeCl_3$ and various concentrations of daphnetin, daphnoretin, apigenin, luteolin, α-tocopherol, and BHT. a = $P < 0.001$; b = $P < 0.01$; c = $P < 0.05$ vs. 18:2 ox (residual linoleic acid).

natural compounds, luteolin, α-tocopherol, and daphnetin exerted a comparable activity at 2.5 µg (8.7, 5.8, and 14.0 nmol, respectively). The addition of EDTA to the system enhanced the activity of all the compounds (data not shown). During the iron-catalyzed oxidation of linoleic acid (Fig. 5), pure compounds were active, giving full protection with a concentration of 1 µg (4.5 nmol) for BHT, 10 µg (56.2 nmol) for daphnetin and luteolin, and 20 µg (46.4 nmol) for α-tocopherol. Table 1 shows the molar concentrations that gave an inhibition of 50% (IC_{50}), calculated from the plot of concentration-dependent residual linoleic acid and HPODE formed. On a molar basis, BHT was the most powerful inhibitor of linoleic acid oxidation in both systems, with an IC_{50} of 0.65 and 1.9 nmol. Daphnetin, although less effective, exerted an activity comparable to luteolin and α-tocopherol during linoleic acid autoxidation and oxidation in the presence of iron. Daphnoretin was again effective only at high concentrations. Apigenin was not active in any treatment. The effectiveness of daphnetin and luteolin was confirmed against lipid peroxidation (15–19). Therefore, the antioxidant activity of the extracts could mainly be due to these molecules, together with α-tocopherol. The use of these *in vitro* systems allows us to discriminate geometrical HPODE isomers formed during linoleic acid oxidation. The ratio of the HPODE formed (*c,t/t,t*) shows hydrogen atom-donating activity (20). The formation of HPODE isomers during the autoxidation process exhibits a paraboloid pattern. Although the shift toward *c,t* isomers disappears with the FA degradation, it is enhanced in the presence of a strong hydrogen atom donator (20). Results obtained during linoleic acid autoxidation are reported in Table 2. The *c,t/t,t* ratio obtained when α-tocopherol was present in the incubation mixture was the only treatment that clearly shifted toward *c,t* isomers. Methanol extracts from *D. gnidium* also shifted the *c,t/t,t* ratio in a positive direction (Table 2). The prevailing mechanism of action of the extracts involves the hydrogen atom-donating activity and is in agreement with our analyses of their composition. In fact, not only α-tocopherol but also daphnetin and luteolin gave a slight shift in the *c,t/t,t* ratio at the highest concentrations (Table 2). *Daphne gnidium* contains a toxic compound, daphnetoxin, found in the bark extract (8,21); however, in the methanol extracts tested this compound was absent. These findings show

TABLE 1
IC_{50} Values (concentration that gives an inhibition of 50%) Calculated from the Plot of Concentration-Dependent Residual Linoleic Acid and Its Hydroperoxides Formed During Oxidation

	Linoleic acid autoxidation	Linoleic acid oxidation in the presence of $FeCl_3$
Compound	IC_{50} (nmol)	IC_{50} (nmol)
Daphnetin	2.2	18.3
Daphnoretin	35.5	155.0
Luteolin	1.4	12.0
Apigenin	>370.1	>370.1
BHT	0.7	1.9
α-Tocopherol	1.7	27.5

TABLE 2
Values of the Ratio of Hydroperoxy-octadecadienoic Acid (HPODE) *c,t/t,t* Isomers Formed During Linoleic Acid Autoxidation in the Presence of the Two Methanol Extracts, Daphnetin, Luteolin, α-Tocopherol, and BHT[a]

	HPODE *c,t*/HPODE *t,t*					
Conc. (µg)	Leaf extract	Stem extract	Daphnetin	Luteolin	α-Tocopherol	BHT
0.1			1.26	1.27	1.26	1.28
0.25			1.29	1.28	1.27	1.32
0.5			1.28	1.26	1.24	3.28
1	1.56	1.19	1.49	2.28	1.20	3.09
2.5	1.27	1.24	2.41	2.10	2.25	3.78
5	1.53	1.22	2.38	2.20	6.35	3.89
10	3.33	1.23	2.65	2.26	6.41	4.79
20	3.99	1.69	2.64	2.51	12.91	4.75
30	5.66	2.12	2.70	2.64	11.55	5.05
50	7.41	2.92	2.72	3.07	13.23	4.52
100	9.89	5.00	2.58	3.71	20.28	5.44
18:2	3.74					
18:2 ox	1.15					

[a]Reference values obtained during linoleic acid autoxidation are also reported. 18:2 ox, residual linoleic acid; *c,cis* configuration; *t, trans* configuration.

that *D. gnidium* extracts not only exhibit medicinal properties but also contain concentrated antioxidant compounds. Further research is needed to investigate the activity and fully characterize the composition of organs of the plant for a possible appropriate use in cosmetic and food products.

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