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Characterization of the Volatile Pattern and Antioxidant Capacity of Essential Oils from Different Species of the Genus *Ocimum*

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The antioxidant capacity of essential oils obtained by steam hydrodistillation from five species of the genus *Ocimum*, namely *Ocimum basilicum var. purpurascens*, *Ocimum basilicum*, *Ocimum gratissimum, Ocimum micranthum*, and *Ocimum tenuiflorum (syn. O. sanctum)*, were evaluated using a high-performance liquid chromatography-based hypoxanthine/xanthine oxidase and the DPPH assays. The yield of oils from the leaves of the five species was variable with the greater amount obtained from *Ocimum gratissimum* (3.5%) and the least from *Ocimum basilicum var. purpurascens* (0.5%). In the hypoxanthine/xanthine oxidase assay, strong antioxidant capacity was evident in all the oils but the greater was shown by that obtained from *Ocimum tenuiflorum (syn. O. sanctum)* ($IC_{50} = 0.46$ μL/mL) compared to *Ocimum basilicum var. purpurascens* ($IC_{50} = 1.84$ μL/mL). Antioxidant capacity was positively correlated ($r = 0.92$, $p < 0.05$) with a high proportion of compounds possessing a phenolic ring such as eugenol, while a strong negative correlation ($r = -0.77$, $p > 0.1$) with other major volatiles was observed. These correlations were confirmed to a large extent in the DPPH assay. The results of a 24 h experiment with *Ocimum tenuiflorum (syn. O. sanctum)* shows that the antioxidant capacity factor (amount of essential oil obtained × free radical scavenging capacity; mg × %/100) reaches a threshold between 10 and 12.00 h, corresponding to maximum sunlight intensity in Brasil and furthermore exhibits a clear diurnal variation. The data generated with *Ocimum* species indicates that essential oils obtained from various herbs and spices may have an important role to play in cancer chemoprevention, functional foods, and in the preservation of pharmacologic products.

KEYWORDS: HPLC; hypoxanthine; mass spectrometry; *Ocimum*; reactive oxygen species; xanthine oxidase

INTRODUCTION

The most widely used synthetic antioxidants used historically in the preservation of foodstuffs such as butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, and tertiary butyl hydroquinone are suspected to cause or promote negative health effects (*1*−*2*). Indeed, they have been replaced in Japan since 1996 by the natural secondary plant metabolite ellagic acid. For this reason, there is a growing interest in replacing synthetic compounds with natural secondary plant metabolites as potential antioxidants. A range of plants has been studied in recent years as potential sources of antioxidants. Among these, many essential oils of aromatic plants and spices have been shown to be effective in retarding the process of lipid peroxidation in oils and fatty foods and have gained the interest of many research groups. Therefore, a systematic examination of antioxidant properties of various plant extracts is extremely important to validate the use of, e.g., essential oils as preservatives in both the food and pharmaceutical industries.

A number of studies on the antioxidant activities of essential oils from various aromatic plants have already shown, e.g., that oils obtained from the genus *Oregano*, rich in thymol and carvacrol, have a considerable antioxidant effect on lipid peroxidation of lard (*3*−*4*).

The genus *Ocimum*, belonging to the family *Labiatae*, comprises 30 species which are found in tropical and subtropical regions. Sweet basil (*Ocimum basilicum L.*) is grown commercially as a cultivated herb in the U.S., the Mediterranean region, and in many other parts of the world. This popular herb is used both as a fresh and dried food spice and also in traditional medicine. The estimated production of basil herb was 2500 tons in 1991 (*5*−*6*) and sweet basil oil, 43 tons in 1991 (*7*).

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On the basis of more than 200 analyses of oils extracted from *O. basilicum*, Lawrence (*8*) delineated four essential oil chemotypes (methyl chavicol, linalool, methyl eugenol, and methyl cinnamate) and also numerous subtypes. According to the biosynthetic origins of the major compounds, they were classified as chemotypes with single or dual biosynthetic pathways. Other chemotypes such as eugenol-rich and thymol-rich *O. gratissimum* (*9*−*13*), sesquiterpenes-rich *O. canum* (*14*), and terpinen-4-ol-rich *O. canum* (*15*) have been reported. However, there is little available published data on the antioxidant potential of essential oils of the *Ocimum* species. Therefore, essential oils of five *Ocimum* species were obtained and antioxidant capacity evaluated in two different in vitro assays. The major pure compounds detected in the oils by gas chromatography mass spectrometry (GC-MS) were also evaluated using the same methods.

MATERIALS AND METHODS

Chemicals. Authentic trans-β-caryophyllene, 1,8-cineole (eucalyptol), eugenol, estragole (methyl chavicol), and linalool were obtained from Extrasynthese (Lyon Nord, Genay, France); $Fe_2Cl_3 \cdot 6H_2O$ from Aldrich, (Steinheim, Germany); *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide and 2,2-diphenyl-1-picrylhydrazil (DPPH) from Sigma Chemie (Deisenhofen, Germany); acetic acid, acetonitrile, dichloromethane, methanol, hypoxanthine, xanthine oxidase, ethylenediamine tetraacetic acid, and salicylic acid from Merck (Darmstadt, Germany); and K_2HPO_4 and KH_2PO_4 from Serva (Heidelberg, Germany). Extra virgin olive oil from Italy (Apulien) was a gift from Zait (67265 Grunstadt, Germany). All solutions were made up in double-distilled water.

Plant Material. Leaves from adult plants of five species of the genus *Ocimum* were collected during the flowering phase (March/April) from the Medicinal Plant Gardens of the Universidade Federal do Ceará (UFC), Fortaleza, Ceará state, Brazil and immediately extracted by steam hydrodistillation. Voucher specimens are deposited at the Herbarium Prisco Bezerra of the UFC, Fortaleza, Brazil.

Steam Hydrodistillation. The fresh leaves of the *Ocimum* species (1 kg) were extracted (1 h) by steam hydrodistillation with hexane as the collecting solvent, immediately after harvesting using a modified Clevenger apparatus to yield the essential oils.

Microwave Hydrodistillation. To assess possible diurnal effects (*16*) of antioxidant capacity, the leaves of *Ocimum* species were collected at 2 h intervals from 6 to 18.00 h on a particular day in high summer. Fresh leaves (50 g) were extracted by hydrodistillation for 8 min using a modified microwave oven method (*17*). The water/oil distillates were collected on dry ice and extracted by partition into dichloromethane.

Gas Chromatography Coupled with Mass Spectrometry (GC-MS). Analyses were performed using a Hewlett-Packard (HP) 5971 mass selective detector coupled to a HP 5890 gas chromatograph. Sample volumes of 1 μL were injected in the split mode (1:10) into the gas chromatograph. Separation of analytes was achieved using a HP dimethylpolysiloxane DB-1 fused-silica capillary column, (30 m $\times$ 0.25 mm i.d., 0.10 μm film thickness). Helium was used as carrier gas with linear velocity of 1 mL/min. The oven temperature program was as follows. Initial temperature 35 °C, 35−180 °C at 4 °C/min, followed by 180−250 °C at 10°C/min. The GC injector temperature was 250 °C; the transfer line temperature was held at 280 °C. The mass spectrometer parameters for EI mode were ion source temperature, 200 °C; electron energy, 70 eV; filament current, 34.6 μA; electron multiplier voltage, 1200 V. Individual components were identified by spectrometric analyses using two computer library MS searches and the aid of Kovat's indices (*18*). Visual mass spectra comparison with data from the literature was used for confirmation (*19*).

Analytical High Performance Liquid Chromatography (HPLC). Reversed-phase analytical HPLC was conducted on a Hewlett-Packard (HP) 1090 liquid chromatograph fitted with a C18 (Latek, Eppelheim, Germany), 25 cm, (5 μm) column, (internal diameter, 4.0 mm). For the separation of individual compounds, the mobile phase consisted of acetic acid (2%) in double-distilled water (A) and methanol (B) utilizing the following gradient over a total run time of 50 min: 95% A for 2 min, 75% A in 8 min, 60% A in 10 min, 50% A in 10 min, and 0% A until completion of the run.

Table 1. Yield of Essential Oils from *Ocimum* Species after Steam Hydrodistillation

Ocimum species	yield (%)	voucher[a] No.
Ocimum gratissimum	3.6	18.671
Ocimum basilicum	3.0	18.670
Ocimum micranthum	2.5	29.315
Ocimum tenuiflorum (syn. O. sanctum)	2.1	IPA-52495
Ocimum basilicum var purpurascens	0.5	18.777

[a] Voucher specimens are deposited at the Herbarium, Prisco Bezerra of the UFC, Fortaleza, Brazil.

Hypoxanthine/Xanthine Oxidase HPLC-Based Assay. The method employed to assay the hydroxyl radical scavenging ability of essential oils obtained from the *Ocimum* species was based on the method of Owen et al. (*20*). The oils and pure compounds were dispersed in the assay buffer at a concentration of 2.0 μL/mL and diluted appropriately (in duplicate) in assay buffer to a final volume of 1.0 mL giving a range of 0.05−2.0 μL/mL, and 10 μL of xanthine oxidase (18 mU), diluted in 3.2 M NH_4SO_4 was added to initiate the reactions. The tubes were incubated for 3 h until reaction completion at 37 °C. After incubation, 20 μL of the reaction mixture was analyzed by HPLC using the mobile phase and conditions described under HPLC. The hydroxylation of salicylic acid and hypoxanthine were monitored at $A = 325$ and $A = 278$ nm, respectively. The amount of dihydroxyphenols (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) produced by hydroxyl radical (HO$^\bullet$) attack on salicylic acid was determined from standard curves of the respective pure dihydroxyphenols.

DPPH Assay. The free radical scavenging capacity of the oils was also determined using the DPPH$^\bullet$ discoloration method (*21*). The oils and pure compounds were diluted in methanol giving a range of 0.05−2.0 μL/mL. The dilutions (20 μL) were placed in a 96-well plate in duplicate. The reaction was initiated by addition of 180 μL DPPH solution (20 μg/mL in methanol). The absorbance was read at 515 nm over 45 min with a universal Micro plate reader (Bio-Tek Instruments, Winooski, VT) compared to duplicate controls containing methanol (20 μL) only. The concentration of DPPH radical was calculated against a standard curve of DPPH (1−100 μg/mL) measured simultaneously.

Statistics. The volume of essential oil or pure compound producing 50% (IC_{50}) inhibition of oxidation or reduction in the hypoxanthine/xanthine oxidase and DPPH assays, respectively, were determined using the Table curve program (Jandel Sciencific, Chicago, IL). The starndard errors at each concentration used were lower than 2% and are therefore not shown in either the tables or figures.

RESULTS

Basic Characteristics of the Oils. The amount of essential oil obtained from the *Ocimum* species was variable (**Table 1**). The greater yield was from *Ocimum gratissimum* (3.5%) and the least from *Ocimum basilicum var. purpurascens* (0.5%). The major volatile components detected and identified by GC-MS was also variable (**Tables 2**−**3**). A major volatile was eugenol, detected in three of the five species (maximum in *Ocimum micranthum* at 64.8%), while estragole (33.1%) was detected in one species only. Other major components detected were trans-β-caryophyllene, 1,8-cineole (eucalyptol), linalool and α-muurolol.

Antioxidant Capacity of the Oils. The greater antioxidant capacity ($IC_{50} = 0.46$ μL) assessed by the HPLC-based hypoxanthine/xanthine oxidase assay was determined (**Table 4**) in the essential oil obtained from *O. tenuiflorum (syn. O. sanctum)* with the least in *O. basilicum var. purpurascens* ($IC_{50} = 1.84$ μL). The concentration-dependent scavenging of reactive oxygen species by the oils is depicted in **Figure 1**.

Table 2. Chemical Composition (%) of Essential Oils of *Ocimum Gratissimum* (I), *Ocimum Micranthum* (II), and *Ocimum Tenuiflorum* (III) as Determined by Gas Chromatography Mass Spectrometry[a]

components	retention index	I %	II %	III %	identification method
α-Pinene	939	0.7	–	–	MS
β-Pinene	979	2.5	–	–	MS
1,8-Cineole	1031	21.6	1.5	–	GC-MS
(*Z*)-Ocimene	1037	4.0	–	–	MS
Linalool	1097	–	0.7	–	GC-MS
Endo-Borneol	1169	–	–	1.0	MS
α-Terineol	1189	0.7	–	–	MS
γ-Elemene	1338	–	1.0	–	MS
Eugenol	1359	54.0	64.8	59.4	GC-MS
(E)-β-Caryophyllene	1419	5.3	14.3	29.4	GC-MS
α-Humulene	1455	0.8	2.3	1.5	MS
allo-Aromadendrene	1460	–	0.7	–	MS
Germacrene D	1485	2.3	–	–	MS
β-Selinene	1490	5.5	1.5	–	MS
α-Selinene	1498	2.6	–	0.7	MS
Bicyclogermacrene	1500	–	8.1	–	MS
Germacrene A	1503	–	2.0	8.1	MS
β-Bisabolene	1509	–	1.1	–	MS
Elemicin	1557	–	2.1	–	MS

[a] GC-MS, identification based on retention time and mass spectra (on a HP dimethylpolysiloxane DB-1 fused silica capillary column) of authentic compounds; MS, tentatively identified on the basis of library searches and Kovat's indices (*18*).

Table 3. Chemical Composition (%) of Essential Oils of *Ocimum Basilicum* ((IV), *Ocimum Basilicum* Var. *Purpurascens* (V) as Determined by Gas Chromatography Mass Spectrometry[a]

components	retention index	IV %	V %	identification method
α-Pinene	927	–	0.6	MS
Camphene	935	0.9	0.4	MS
Sabinene	955	1.6	–	MS
β-Pinene	958	–	1.3	MS
p-Cymene	1001	–	0.5	MS
1,8-Cineole	1004	11.0	5.0	GC-MS
Limonene	1008	0.7	1.0	MS
γ-Terpinene	1038	–	0.3	MS
Linalool	1091	42.5	39.3	GC-MS
Camphor	1107	0.9	0.9	MS
Borneol	1139	–	0.6	MS
Terpinen-4-ol	1155	3.3	1.8	MS
Estragole	1187	33.1	1.9	GC-MS
Octyl acetate	1204	–	2.3	MS
Bornyl acetate	1271	–	0.9	MS
δ-Elemene	1343	–	3.4	MS
α-Copaene	1377	–	0.6	MS
β-Bourbonene	1383	–	0.5	MS
β-Cubebene	1384	–	2.0	MS
β-Elemene	1393	–	0.8	MS
(*E*)-β-Caryophyllene	1416	–	0.8	GC-MS
(*E*)-α-Bergamotene	1442	1.5	4.0	MS
α-Humulene	1449	–	1.6	MS
α-Muurolene	1473	0.8	–	MS
Bicyclogermacrene	1491	–	0.7	MS
β-Eudesmol	1630	–	0.5	MS
Germacrene A	1499	–	1.0	MS
δ-Guaiene	1503	–	0.4	MS
γ-Cadinene	1512	1.2	7.7	MS
Humulene epoxide II	1586	–	1.5	MS
1-Epi-cubenol	1600	–	2.3	MS
α-Muurolol	1627	2.5	11.0	MS

[a] GC-MS, identification based on retention time and mass spectra (on a HP dimethylpolysiloxane DB-1 fused silica capillary column) of authentic compounds; MS, tentatively identified on the basis of library searches and Kovat's indices (*18*).

The antioxidant capacity of the oils correlated positively ($r = 0.92$, $p < 0.05$) with the major proportion of volatile components in the oils and to compounds possessing a phenolic ring such as eugenol ($r = 0.92$, $p < 0.05$), whereas a strong negative correlation ($r = -0.77$, $p > 0.1$) with other volatiles was observed. It should also be noted, however, that the essential oil obtained from *O. basilicum var. purpurascens* containing linalool as the major component but lacking a phenol ring also showed a relatively strong antioxidant capacity. Studies with available commercial compounds (**Figure 2**), confirmed these results in this assay in that linalool which was present as a major component of both oils with the lesser IC_{50} was in pure form, equally as potent as eugenol. Three of the oils also inhibited xanthine oxidase significantly (**Figure 3**). However, no logical structure correlations could be observed in that none of the pure compounds studied inhibited xanthine oxidase. Pure extra virgin olive, sesame, and corn oils were totally ineffective in comparison to the essential oils obtained from the *Ocimum* species studied in terms of antioxidant capacity.

Table 4. Inhibitory Concentration (IC_{50}) in the HPLC-Based Hypoxanthine/Xanthine Oxidase and DPPH Assays of Essential Oils Extracted by Steam Hydrodistillation from *Ocimum* Species

Ocimum species	assay IC_{50} (μL/mL) HX/XO	DPPH•
Ocimum basilicum var. purpurascens	1.84	>2.0
Ocimum basilicum	1.42	>2.0
Ocimum gratissimum	0.94	0.29
Ocimum micranthum	0.82	0.41
Ocimum tenuiflorum (syn. O. sanctum)	0.46	0.26

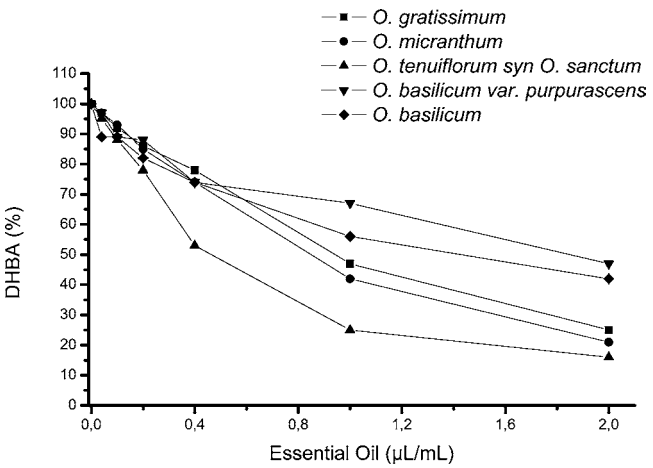

Figure 1. Dose-dependent inhibition of reactive oxygen species attack on salicylic acid by essential oils of *Ocimum* species in the HPLC-based hypoxanthine/xanthine oxidase assay (mean of 2 assays).

The results of the HPLC-based hypoxanthine/xanthine oxidase assay were largely confirmed by the DPPH assay (**Table 4**); however, linalool, which displays a relatively strong antioxidant capacity in the former, has no such effect in the latter assay, reflecting its susceptibility to attack by HO• but its inability to reduce DPPH•. In the DPPH assay, of the pure compounds tested only eugenol ($IC_{50} = 0.04$ μL/mL) displayed potent free radical scavenging capacity.

Given that the production of secondary plant metabolites is mainly related to the preservation of the organism, it was of interest to determine whether the production of antioxidant volatile compounds showed any temporal variation in *Ocimum* species. The results of a 24 h experiment with *Ocimum tenuiflorum (*syn. *O. sanctum)* are given in **Figure 4**, using the HPLC-based hypoxanthine/xanthine oxidase assay, and shows that the antioxidant capacity factor (amount of essential oil

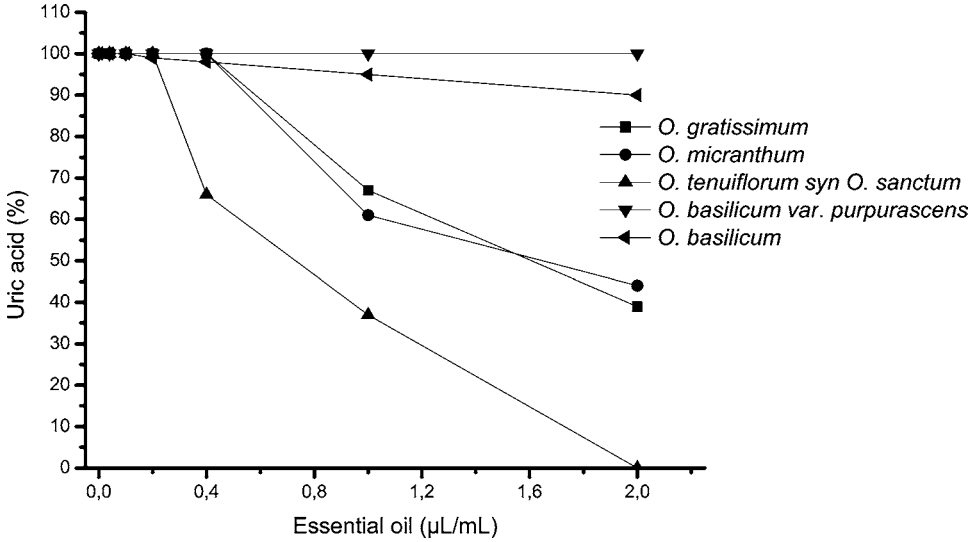

Figure 2. Dose-dependent inhibition of xanthine oxidase by essential oils of *Ocimum* species in the HPLC-based hypoxanthine/xanthine oxidase assay (mean of 2 assays).

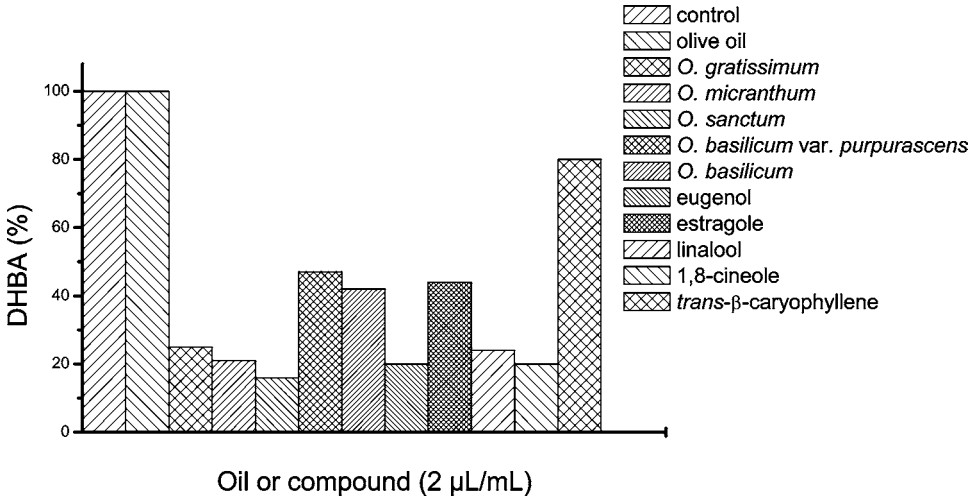

Figure 3. Comparison of the inhibitory effect of essential oils with olive oil and pure compounds against reactive oxygen species attack on salicylic acid in the HPLC-based hypoxanthine/xanthine oxidase assay (mean of 2 assays).

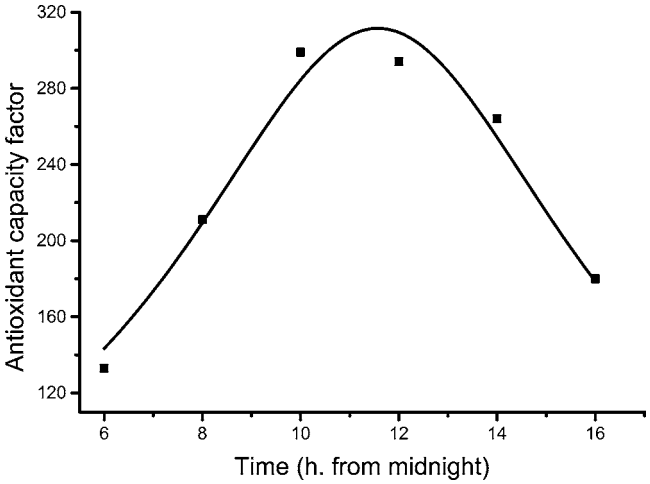

Figure 4. Diurnal antioxidant capacity factors of essential oil from the leaves (50 g) of *Ocimum sanctum* at different time points during the day (mean of 2 assays).

obtained × free radical scavenging capacity; mg × %/100) reaches a threshold between 10 and 12.00 h, corresponding to maximum sunlight intensity in Brazil, and furthermore exhibits a clear diurnal variation. However, the antioxidant capacity of the oils was very similar at each time point, which is not surprising considering that each oil contained virtually identical amounts of eugenol (95%).

DISCUSSION

The results of this study show that the yield of essential oils from *Ocimum* species is variable, with *O. gratissimum* providing over seven times more than *O. basilicum* var *purpurscens*. The IC_{50} values measured in the hypoxanhine/xanthine assay for extracts of each *Ocimum* species was extremely low, especially that of *O. tenuiflorum (*syn. *O. sanctum)*, even in comparison with other polyphenol-rich oils, e.g., olive and sesame (*20*). The antioxidant capacity of the oils, however, is not clearly related to the proportion and profile of secondary plant compounds, and a phenolic ring in the structure based on the studies with pure compounds does not appear to be a major determinant with regard to scavenging of the hydroxyl radical. Although a 4-fold variation in antioxidant capacity was observed, the low IC_{50} values of all oils probably lends futility in searching for correlations with particular constituents. The take home message is that essential oils (as is) of *Ocimum* species display far greater antioxidant capacities compared to seasoning oils such as olive

(*20*) and sesame which also contain considerable amounts of phenolic compounds.

Furthermore, while diversity is necessary, an increase in the horticulure of *O. tenuiflorum (syn. O. sanctum)* would appear to be advantageous in terms of future applications for cancer chemoprevention and incorporation into functional foods and pharmaceutical products. Also, benefits may accrue by the utilization of technologic processes or genetic manipulation to increase the yield of oil from this species. Furthermore, harvesting between 10 and 12.00 h may yield a higher content of antioxidant secondary plant substances in the oil because the antioxidant capacity at these time points is greater, presumably to scavenge the reactive oxygen species generated by ultraviolet light at maximum sunlight intensity. The oils are currently undergoing a battery of further in-vitro tests (*22*) to clarify their cancer chemopreventive potential and preservation of pharmaceutical products and validity as food additives.

ABBREVIATIONS USED

DHBA, dihydroxybenzoic acids; DPPH, 2,2-diphenyl-1-picrylhydrazil; GC-MS, gas-chromatography mass spectrometry; HP, Hewlett-Packard; HPLC, high performance liquid chromatography; UV, ultraviolet; ROS, reactive oxygen species.

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