JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Composition of the Volatile Fraction of *Ocotea bofo* Kunth (Lauraceae) Calyces by GC-MS and NMR Fingerprinting and Its Antimicrobial and Antioxidant Activity

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The chemical composition of the essential oil obtained by steam distillation of the floral calyces of *Ocotea bofo* Kunth (Lauraceae) was studied by means of GC, GC-MS, and ^{1}H, ^{13}C, and bidimensional NMR (COSY, HSQC, HMBC). Twenty-five constituents were identified, and estragole (48.7%), α-phellandrene (19.6%) and sabinene (10.4%) were found to be the major components. Antimicrobial activity against six aerobic bacteria and five yeasts and antioxidant activity performed by photochemiluminescence (PCL), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and β-carotene bleaching assays are reported. The oil showed fair inhibiting properties against bacteria and a good inhibition against most yeasts. Its radical scavenging and chain-breaking antioxidant properties were comparable to or better than those provided by synthetic controls. Particular emphasis has been given to the use of NMR as a fast and reliable tool to discriminate *O. bofo* essential oil from other commercial anethole- and estragole-rich oils, namely, *Illicium verum*, *Foeniculum vulgare*, and *Artemisia dracunculus*.

KEYWORDS: ***Ocotea bofo*; essential oil; antimicrobial; antioxidant; NMR fingerprinting**

INTRODUCTION

The *Ocotea* genus includes more than 350 tropical and subtropical species, mainly distributed in the Americas and in southern Africa, and constitutes a large source of spicy essential oils, including those of *O. pretiosa* and *O. cymbarum*, which have a renewed market position (*1*). Moreover, many *Ocotea* species have caught the attention of phytochemists and pharmacologists for the presence of unusual lignans, neolignans, and alkaloids (*2*−*4*), and some of them (*O. bullata* and *O. duckei*) are reputed traditional remedies in southern Africa (*4*) or possess a proven pharmacotherapic action (*5*, *6*). The high degree of chemodiversity in the essential oils from Lauraceae members is widely recognized, and within the *Ocotea* genus and the related *Persea* and *Nectandra* genera many unique components such as asaricin and caparratriene have been reported (*7*−*10*). Some of them have shown antifungal and antimicrobial activities (*7*). Moreover, phenylpropanoids are often encountered, and their presence is often related to good antioxidant properties (*11*).

Ocotea bofo Kunth (Lauraceae) is a medium-sized tree with simple, longish, and acuminate leaves with pink pedicels, small milky flowers collected in hairy cymes, and persistent reddish calyces. The fruit is a small red berry, and the whole plant possesses a strong aromatic smell. Within its distributional area, *O. bofo* is known as "anis de arbol" (Ecuador), "moena rosa" (northern Peru), or "pau de quiabo" (Brazil) (*12*). The tree is frequently found in eastern Amazonian lowlands (*13*, *14*), where its woody calyces collected from mature fruits are traditionally used to aromatize infusions by Shuar and Achuar ethnic groups. As its trivial name suggests, the plant possesses a strong anise-like aroma and thus may represent a potential aniseed, fennel, or tarragon substitute or adulterant.

Different chromatographic and spectroscopic methodologies are currently available for essential oil fingerprinting, both for quality definition and for fraud detection. The characterization of complex mixtures of volatile compounds is in fact usually achieved through high-resolution chromatography techniques such as high-performance thin-layer chromatography (HPTLC) and capillary gas chromatography (GC) or hyphenated tech-

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10.1021/jf0605493 CCC: $33.50
Published on Web 09/13/2006

niques such as mass spectrometry (MS), enantiomeric and comprehensive two-dimensional gas chromatography−mass spectrometry (enantio-GC; GC/GC-MS) (*15*−*17*). During the past decade, high-resolution nuclear magnetic resonance (NMR) has emerged as a powerful tool for the fingerprinting of crude or partially refined natural extracts, including terpenes, sesquiterpenes, phenylpropanoids, and other volatile compounds, and to assess the quality of raw materials aimed for food, herbal, and pharmaceutical industries (*18*, *19*). Such an approach appears to be very fast and reliable and allows also the detection of eventual nonvolatile adulterants. However, its routine use in essential oils analysis (in particular to discriminate essential oils with similar composition) is not well established at present and specific and comparative data are scarcely available, with rare exceptions (*19*).

In this paper, we report for the first time a detailed study on the chemical characterization and the food-related preservative properties (antimicrobial, antioxidant) of the essential oil obtained from *O. bofo* calyces, a scarcely studied Amazonian spice. A complete comparison with commercial oils rich in anethole isomers (*Illicium verum*, *Artemisia dracunculus*, and *Foeniculum vulgare*) is also provided, with the aim to make available a basic set of data useful to discriminate *O. bofo*, *I. verum*, *A. dracunculus*, and *F. vulgare* oils. In this regard, particular emphasis has been given to the correlation between chromatography and ^{1}H, ^{13}C, and multidimensional NMR and to the identification of characteristic fingerprinting markers.

MATERIALS AND METHODS

Chemicals. All of the chemicals employed for antioxidant assays were purchased from Sigma-Aldrich Italy or Analytikjena, Jena, Germany, whereas microbial culture media (TSA, NA) were from Oxoid Italia (Garbagnate, Italy) and positive control antibioticals Ampicillin and Fluconazole were from Sigma-Aldrich and Pfizer, respectively. Compounds used as references for GC, GC-MS, and NMR (namely, estragole, *trans*-anethole, α-phellandrene, limonene, *cis*-*Z*-ocimene, linalool, and *p*-anisaldehyde) were from Sigma-Aldrich (Milan, Italy), from both "General" and "Flavors and Fragrances" catalogs. Water was purified by a Milli-Q_{plus} 185 system from Millipore (Milford, MA). Commercial *I. verum* essential oil (Biokyma, Anghiari, Italy) was obtained from a local grocery. *F. vulgare* and *A. dracunculus* essential oils were from Pam'Innov, Le Chaffaut-Saint-Jurson, Provence, France, and came from locally cultivated plants. *Thymus vulgaris* essential oil, thymol chemotype, was purchased from Extrasynthese (Genay, France).

Plant Material. *O. bofo* dried calyces were supplied by Fundacion Chankuap' (Macas, Ecuador) collected in January 2002 from wild trees on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77° 15″ W, 2° 35″ S) and positively identified by the National Herbary of Pontificia Universidad Catolica del Ecuador. A dried specimen of the spice was deposited at the Dipartimento delle Risorse Naturali e Culturali, University of Ferrara, Code N. OBF001.

Essential Oil Isolation. The finely ground calyces (500 g) were hydrodistilled according to European Pharmacopoeia methods, and 3.9 mL of a pale yellow oil was obtained. Essential oil yield was determined on a volume to dry weight basis as 0.78 ± 0.21%. The values for essential oil yield of the three replications were averaged and standard deviations calculated. The essential oil samples were stored in glass vials with Teflon-sealed caps at 2 ± 0.5 °C in the absence of light.

Gas Chromatography. Essential oil samples were analyzed and the relative peak areas for individual constituents averaged. The relative percentages were determined using a Fisons (Rodano, Milano, Italy) 9130-9000 series gas chromatograph equipped with a Fisons EL980 processor, a FID detector, and a Mega SE52 (Mega, Legnano, Italy) poly-5% diphenyl−95%-dimethyl-siloxane bonded phase column (i.d., 0.32 mm; length, 30 m; film thickness, 0.15 μm). Operating conditions were as follows: injector temperature, 280 °C; FID temperature, 280 °C; carrier (helium) flow rate, 2 mL/min; and split ratio, 1:40. Oven temperature was initially 45 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min, and finally held at that temperature for 10 min. One microliter of each sample dissolved in CH_2Cl_2 was injected. The percentage composition of the oils was computed by the normalization method from the GC peak areas, without using correction factors.

Gas Chromatography−Mass Spectrometry Analysis. Essential oil constituents were then analyzed by a Hewlett-Packard HP5890 series II plus gas chromatograph equipped with an HPMS 5989b mass spectrometer using electron impact and hooked to the NBS75K library. The constituents of the volatile oils were identified by comparing their GC retention times, KI, and MS fragmentation patterns with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns and retention indices with the above-mentioned mass spectra libraries and with those in the literature (*20*). The GC conditions were the same as reported for GC analysis, and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 40 μA; scan rate, 1 scan/s; mass range, 35−300 Da; ion source temperature, 200 °C. A mixture of aliphatic hydrocarbons (C_8−C_{24}) in hexane was injected under the above temperature program to calculate the retention indices using the generalized equation by Van del Dool and Kartz (*21*).

NMR Spectroscopy. The ^{1}H, ^{1}H−^{1}H COSY, HSQC, and HMBC spectra were recorded on a Varian Gemini-400 spectrometer operating at 399.97 MHz and at a temperature of 303 K. Essential oils or reference compounds (25 mg/1.0 mL) were dissolved in deuterated chloroform into a 5 mm NMR tube, and the solvent signal was used for spectral calibration (^{1}H 7.26 ppm). ^{1}H spectra were run using a standard pulse sequence "s2pul", with 45.0 degrees pulse, 3.00 s acquisition time, 8 repetitions, 4000 Hz spectral width, and 0.33 Hz FID resolution. The ^{13}C NMR spectra were recorded at 75.46 MHz and at a temperature of 303 K. The essential oils (120 mg/1 mL) were dissolved in deuterated chloroform. The same spectral calibration of ^{1}H NMR experiments was used. The time domain size was 16 K, the spectral width 20000 Hz, and FID resolution 1.24 Hz. ^{1}H−^{1}H COSY experiments were acquired using a gradient-selected COSY (gCOSY), with a 1.000 s relaxation delay, a 0.150 s acquisition time, a single scan, and 256 increments, 4000 Hz in both dimensions for spectral width, 6.7 (F2) and 15.6 Hz (F1) FID resolution. For the gHSQC experiment the ^{13}C GARP-1 decoupled spectrum was recorded at 100.58 MHz with a power of 50 dB on during acquisition and off during delay; the parameters used were a 1.000 s relaxation delay, 0.150 s acquisition time, 16 repetitions and 256 increments, 4000 Hz (^{1}H) and 17094 Hz (^{13}C) for spectral width, and 6.7 Hz (F2) and 66.8 Hz (F1) FID resolution. HMBC experiments were acquired using a 1.000 s relaxation delay, a 0.150 s acquisition time, 32 repetitions and 256 increments, 4000 Hz (^{1}H) and 20120 Hz (^{13}C) for spectral width and 6.7 Hz (F2) and 78.6 Hz (F1) FID resolution.

Characteristic resonances of ^{13}C and ^{1}H NMR spectra for estragole, *trans*-anethole, α-phellandrene, limonene, *cis*-*Z*-ocimene, linalool, and *p*-anisaldehyde were detected according to literature data (*19*, *22*) and by comparison with chemical shifts observed for single constituent solutions. ^{13}C and ^{1}H NMR signals and their correlations for fenchone and α-pinene were detected according to literature data (*19*).

Biological Activities. All of the tested biological activities on *O. bofo* essential oil were compared to those achieved with the commercial essential oil of *T. vulgaris*, in order to have references with an essential oil reputed for its antioxidant, antifungal, and antibacterial properties (*1*). All of the data collected for each assay are the average of three determinations of three independent experiments.

Free Radical Scavenging Activity: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Test. The free radical scavenging activity of essential oils was determined according to the procedure of Choi et al. (*23*). An aliquot of essential oil (10 μL) was mixed with 900 μL of 100 mM Tris-HCl buffer (pH 7.4), 40 μL of ethanol, and 50 μL of 0.5% (w/w) Tween 20 solution. One milliliter of 0.5 mM DPPH ethanol solution was then added to the mixture. Tween 20 was used as an oil-in-water emulsifier. The mixture was shaken vigorously and immediately placed in a UV−vis spectrophotometer (ThermoSpectronic Helios γ, Cambridge, U.K.), and the absorbance at 517 nm was monitored for 70

Table 1. Constituents of Essential Oils of *O. bofo*, *A. dracunculus*, *F. vulgare*, and *I. verum*

				RA[c]			
no.	compound[a]	ID method	KI-polar[b]	*F. vulgare*	*A. dracunculus*	*I. verum*	*O. bofo*
1	α-thujene[d]	GC-MS, NMR	931				0.10
2	α-pinene[d]	GC-MS, NMR	938	0.81	1.32	0.42	0.42
3	camphene[d]	GC-MS, NMR	954		0.10		
4	sabinene[d]	GC-MS, NMR	976	0.37			10.4
5	β-pinene[d]	GC-MS, NMR	979		0.32		
6	β-myrcene[d]	GC-MS, NMR	990	0.14	0.22	0.11	1.57
7	α-phellandrene [d]	GC-MS, NMR	1004	0.05		0.37	19.62
8	α-terpinene[d]	GC-MS, NMR	1015				0.96
9	*p*-cymene[d]	GC-MS, NMR	1027	1.21	0.06	0.11	4.84
10	β-phellandrene[e]	GC-MS, NMR	1030			0.77	
11	limonene[e]	GC-MS, NMR	1030	5.67	4.60		5.00
12	*cis* ocimene[e]	GC-MS, NMR	1037	0.15	8.60		1.81
13	*trans* ocimene[d]	GC-MS, NMR	1051		9.41		0.21
14	γ-terpinene[d]	GC-MS, NMR	1059	0.60			0.29
15	fenchone[d]	GC-MS, NMR	1087	2.82			
16	linalool[d]	GC-MS, NMR	1096			2.34	
17	*allo*-ocimene[d]	GC-MS, NMR	1143	0.24	0.72	-	0.32
18	camphor[d]	GC-MS, NMR	1146	0.12			
19	terpinen-4-ol[d]	GC-MS, NMR	1178	0.06		0.32	0.15
20	α-terpineol[d]	GC-MS, NMR	1183			0.28	
21	*iso*-menthol[d]	GC-MS, NMR	1188		0.34		
22	estragole[e]	GC-MS, NMR	1198	4.56	70.12	6.58	48.71
23	fenchyl acetate[d]	GC-MS, NMR	1233	0.05			
24	*p*-anisaldehyde[e]	GC-MS, NMR	1249	3.99		1.77	
25	anethole[e]	GC-MS, NMR	1253	75.83	0.10	78.8	0.20
26	isobornyl acetate[d]	GC-MS, NMR	1287		0.38		
27	δ-elemene[d]	GC-MS, NMR	1338				0.89
28	eugenol[d]	GC-MS, NMR	1359		0.21		
29	α-copaene[d]	GC-MS, NMR	1377				0.12
30	*p*-acetonylanisole[d]	GC-MS, NMR	1381				
31	β-elemene[d]	GC-MS, NMR	1391				0.11
32	methyleugenol[d]	GC-MS, NMR	1404		1.18		0.08
33	4-methoxyphenylacetone[d]	GC-MS, NMR	1411	1.97		0.41	
34	caryophyllene[d]	GC-MS, NMR	1419		0.16		0.87
35	β-copaene[d]	GC-MS, NMR	1432			0.53	0.05
36	γ-elemene[d]	GC-MS, NMR	1435				0.05
37	α-humulene[d]	GC-MS, NMR	1437				0.14
38	*trans*-bergamotene[d]	GC-MS, NMR	1454			0.47	
39	germacrene D[d]	GC-MS, NMR	1485				0.06
40	β-selinene[d]	GC-MS, NMR	1490				0.23
41	*cis*-nerolidol[d]	GC-MS, NMR	1501			0.16	
42	bicyclogermacrene[d]	GC-MS, NMR	1532				0.56
43	cinnamaldehyde, *p*-methoxy-[d]	GC-MS, NMR	1567		0.12		
44	foeniculin[d]	GC-MS, NMR	1576			2.25	
45	spathulenol[d]	GC-MS, NMR	1678		0.34		
46	hexatriacontane[d]	GC-MS, NMR	3600			1.18	
	total			98.64	98.49	96.87	97.76

[a] Compounds are listed in order of elution from an SE-52 column. [b] Retention indices calculated on an SE52 polar column. [c] Relative area percentage (peak area relative to total peak area percent, calculated on an SE52 polar column. [d] Identified on the basis of comparison with MS database spectra, retention indices, and complete mono/bidimensional NMR spectra interpretation. [e] Identified on the basis of comparison with MS database spectra, retention indices, mono/bidimensional NMR spectra interpretation comapred with those of pure reference compounds.

min, until the reaction reached a plateau. A blank was assessed as the solution assay described above without the essential oils, instead of which distilled water was employed. Trolox (1 mM) was used as a positive control. The radical scavenging activities of each sample were calculated according to the following formula for inhibition percentage (Ip) of DPPH: IpDPPH % = $(A_B - A_A)/A_B \times 100$, where A_B and A_A are the absorbance values of the blank sample and of the test sample, respectively, after 70 min.

Antioxidant Activity: β-Carotene Bleaching Test. The antioxidant activity of essential oils was determined using a β-carotene bleaching test (*24*). Approximately 10 mg of β-carotene (type I synthetic) was dissolved in 10 mL of $CHCl_3$, and 0.2 mL of the solution was pipetted into a flask containing a boiling mixture of 20 mg of linoleic acid and 200 mg of Tween 40. $CHCl_3$ was removed using a rotary evaporator (Büchi 461) at 40 °C for 5 min, and 50 mL of distilled water was slowly added to the residue under vigorous agitation, to form an emulsion. Five milliliters of the emulsion was added to a tube containing 0.2 mL of the essential oils solution prepared according to the method of Choi et al. (*23*), and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tube was placed in a water bath at 50 °C, and the oxidation of the emulsion was monitored spectophotometrically by measuring absorbance at 470 nm over a 60 min period. Positive control consisted of butylated hydroxyanisole (BHA), whereas negative control consisted of test emulsion in which the essential oil was substituted by equal amounts of distilled water. The antioxidant activity was expressed as inhibition percentage with reference to the control after a 60 min incubation using the following equation: $AA = 100(DR_C - DR_S)/DR_C$, where AA is the antioxidant activitt, DR_C si the degradation rate of the control = $[\ln(a/b)/60]$, DR_S is the degradation rate in the presence of the sample = $[\ln(a/b)/60]$, a is the absorbance at time 0, and b is the absorbance at 60 min.

Photochemiluminescence (PCL). The luminol PCL assay was carried out according to the procedure described by Popov and Lewin (*25*) and adapting the standard protocol. The essential oils were measured in the Photochem with the ACL kit (Analytikjena, Jena,

Table 2. Resonance Assignments and Chemical Shifts of Constituents Identified in Essential Oils of *O. bofo*, *A. dracunclus*, *F. vulgare*, and *I. verum* in 400 MHz ^{1}H and ^{13}C NMR Spectra

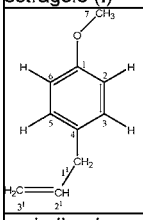
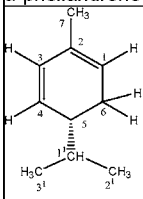
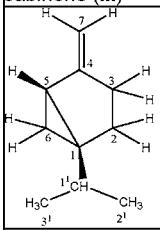
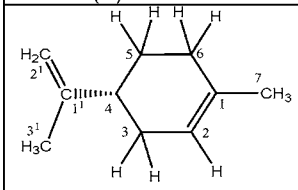
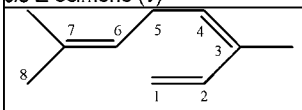
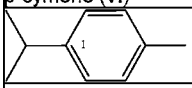
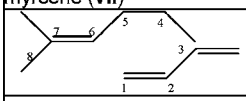
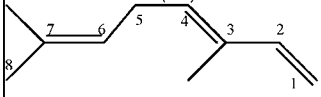
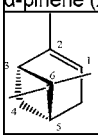
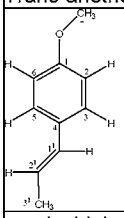
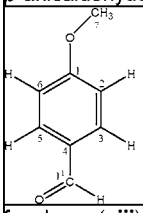
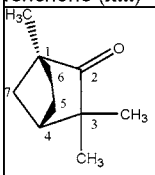
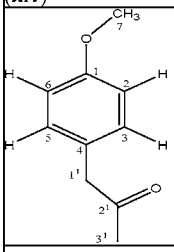
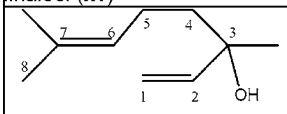
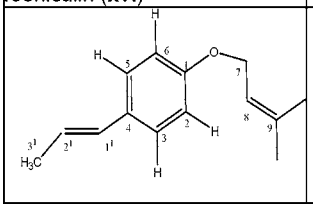
Coumpound	***H[1] shift (ppm) and multiplicity[a]***	***Assignment***	***Observed***	***^{13}C shift (ppm)***
Ocotea bofo				
estragole (**i**)	3.33 (d, 2H, j = 6.8 Hz)	$H_{1'}$	1D, COSY, HSQC, HMBC	39.3
	3.79 (s, 3H)	H_7	1D, HSQC	55.2
	5.04 (ψdd, 1H, j = 10.2, 1.9 Hz)	$H_{3'}$ *trans*[b]	1D, COSY, HSQC, HMBC	115.8
	5.05 (ψddt, 1H, j = 16.8, 1.9,1.5 Hz)	$H_{3'}$ *cis*[b]	1D, COSY, HSQC, HMBC	115.8
	5.95 (ddt, 1H, j = 16.8, 10.5, 6.6 Hz)	$H_{2'}$	1D, COSY, HSQC, HMBC	137.8
	6.84 (ψdt, 2H, j = 8.7, 2.9, 2.2 Hz)	H_2, H_6	1D, COSY, HSQC	113.8
	7.11 (d, 2H, j = 8.7 Hz)	H_3, H_5	1D, COSY, HSQC	129.5
		C_4	1D, HMBC	132.0
		C_1	1D, HMBC	157.9
α-phellandrene (**ii**)	0.89-0.91 (2d, 6H, j = 4.4 Hz)	$H_{2'}$, $H_{3'}$	1D, HSQC	19.8
	1.68 (m,1H)	$H_{1'}$	1D, COSY, HSQC, HMBC	31.2
	1.74 (s, 3H)	H_7	1D, HSQC	21.0
	1.95-2.15 (m, 2H)	H_6	1D, COSY, HSQC, HMBC	25.8
	2.05 (m,1H)	H_5	1D, COSY, HSQC, HMBC	39.6
	5.46 (m, 1H)	H_1	1D, COSY, HSQC, HMBC	120.4
	5.71 (dd, 1H, j = 10.1, 1.7 Hz)	H_4	1D, COSY, HSQC, HMBC	130.1
	5.78 (dt, 1H, j = 10.1, 1.7 Hz)	H_3	1D, COSY, HSQC, HMBC	127.9
		C_2	1D, HMBC	131.1
sabinene (**iii**)	0.66 (d, 2H, j = 5.1 Hz)	H_6	1D, COSY, HSQC	16.0
	0.87-0.94 (2d, 6H, j = 6.8 Hz)	$H_{2'}$, $H_{3'}$	1D, COSY, HSQC	19.8
	1.49 (sept, 1H, j = 6.8 Hz)	$H_{1'}$	1D, COSY, HSQC, HMBC	32.6
	4.61 (s, 1H)	H_7 *trans*[b]	1D, COSY, HSQC, HMBC	101.5
	4.80 (s, 1H)	H_7 *cis*[b]	1D, COSY, HSQC, HMBC	101.5
		C_1	1D, HMBC	37.0
		C_4	1D, HMBC	154.4
limonene (**iv**)	1.67 (s, 3H)	H_7	1D, HSQC, HMBC, COSY	23.5
	1.75 (s, 3H)	$H_{3'}$	1D, HSQC, HMBC, COSY	20.8
	4.70 (m, 2H)	$H_{2'}$	1D, HSQC, HMBC, COSY	108.6
	5.42 (m, 1H)	H_2	1D, HSQC, HMBC, COSY	120.6
		C_4	1D	41.5
		methylene group	1D	30.7
		methylene group	1D	30.5
		methylene group	1D	27.9
		$C_{1'}$	1D, HMBC	150.5
cis-Z-ocimene (**v**)	1.67 (s, 3H) [c]	methyl group	1D, HSQC	17.7
	1.71 (s, 3H) [c]	methyl group	1D, HSQC	25.5
	1.82 (s, 3H) [c]	H_3	1D, HSQC	19.6
	2.84 (t, 2H, j = 5.0 Hz)	H_5	1D, HSQC	26.5
	5.24 (d, 1H, j = 16.8 Hz) [c]	H_1 *cis*[b]	1D, HSQC	113.5
	5.37 (t, 1H, j = 7.6 Hz)	H_4	1D, HSQC	129.6
	6.82 (d, 1H, j = 10.7 Hz)	H_2	1D, HSQC	133.4
		C_6	1D	123.3
p-cymene (**vi**)	1.27 (d, 6H, j = 6.8 Hz)	2 CH_3 isopropyl	1D, HSQC, HMBC, COSY	23.9
	2.32 (s, 3H)	CH_3	1D, HSQC, HMBC	20.9
	2.87 (sept, 1H, j = 6.8 Hz)	CH isopropyl	1D, HSQC, HMBC, COSY	33.8
		C_1	1D, HMBC	145.9
myrcene (**vii**)	5.25 (d, 1H, j = 17.5 Hz)	H_1 *cis*[b]	1D	
	6.38 (dd, 1H, j = 17.5, 10.7 Hz)	H_2	1D, HSQC	139.0
Artemisia dracunculus				
trans-E-ocimene (viii)	2.87 (m, 2H)	H_5	1D, HSQC	27.3
		methyl group	1D	11.5
	4.93 (d, 1H, j = 10.7 Hz)	H_1 *trans*[b]	1D, HSQC	110.5
	5.45 (t, 1H, j = 7.6 Hz)	H_4	1D, HSQC	131.7
	6.40 (dd, 1H, j = 17.6, 10.7 Hz)	H_2	1D, HSQC	141.4
		C_6	1D	122.9
methyl eugenol (**ix**)	3.85 (2s, 6H)	OCH_3	1D, HSQC	56.1
α-pinene (**x**)	0.85 (s, 3H)	methyl group	1D, HSQC	20.6
	1.28 (s, 3H)	methyl group	1D, HSQC	26.2
	1.65 (s, 1H)	CH	1D, HSQC	23.2

Table 2. (Continued)

Coumpound	*H^1 shift (ppm) and multiplicity*[a]	*Assignment*	*Observed*	*^{13}C shift (ppm)*
Foeniculum vulgare				
Trans-anethole (**xi**)	1.87 (dd, 3H, j = 6.7, 1.5 Hz)	$H_{3'}$	1D, HSQC	18.2
	3.79 (s, 3H)	H_7	1D, HSQC	55.2
	6.10 (dq, 1H, j = 15.8, 6.7 Hz)	$H_{2'}$	1D, HSQC	123.5
	6.35 (dq, 1H, j = 15.8, 1.5 Hz)	$H_{1'}$	1D, HSQC	130.4
	6.84 (ψdt, 2H, j = 8.7, 3.0, 1.9 Hz)	H_2, H_6	1D, HSQC	113.8
	7.25 (ψdt, 2H, j = 8.7, 3.0, 1.9 Hz)	H_3, H_5	1D, HSQC	126.8
		C_1	1D	158.6
		C_4	1D	130.8
p-anisaldehyde (**xii**)	3.84 (s, 3H)	H_7	1D, HSQC	55.4
	6.97 (ψdt, 2H, j = 8.5, 2.7, 2.2 Hz)	H_2, H_6	1D, HSQC	114.5
	7.86 (ψdt, 2H, j = 8.5, 2.7, 2.2 Hz)	H_3, H_5	1D, HSQC	131.8
	9.90 (s, 1H)	$H_{1'}$	1D, HSQC	190.7
		C_1	1D	164.6
fenchone (**xiii**)	1.07 (s, 3H)	methyl group (3)	1D, HSQC	21.5
	1.07 (s, 3H)	methyl group (3)	1D, HSQC	23.2
	1.19 (s, 3H)	methyl group (1)	1D, HSQC	14.6
	1.44 (d, 1H, j = 4.9 Hz)	H_6	1D, HSQC	31.8
	2.16 (m, 1H)	H_4	1D, HSQC	45.1
		C_2	1D	223.0
		C_1	1D	54.1
		C_3	1D	47.3
		C_7	1D	41.7
4-methoxyphenylacetone (**xiv**)	3.61 (s, 2H)	$H_{1'}$	1D, HSQC	50.1
	6.89 (m, 2H)	H_2, H_6	1D, HSQC	114.5
	7.16 (d, 2H, j = 8.5 Hz)	H_3, H_5	1D, HSQC	126.3
		$C_{2'}$	1D	207.0
Illicium verum				
linalool (**xv**)	1.31 (s, 3H)	methyl group (3)	1D, HSQC	27.8
	1.59 (m, 2H)	H_4	1D, HSQC	42.2
	1.63 (s, 3H)	methyl group (8)	1D, HSQC	17.7
	1.71 (s, 3H)	methyl group (8)	1D, HSQC	25.7
	2.05 (m, 2H)	H_5	1D, HSQC	22.85
	5.25 (dd, 1H, j = 17.3, 1.3 Hz)	H_1 *cis*[d]	1D, HSQC	111.7
		C_2	1D	145.2
foeniculin (**xvi**)	1.59 (s, 3H)	methyl group (9)	1D	
	4.52 (d, 2H, j = 8.6 Hz)	H_7	1D	

[a] *s*, singlet; *d*, doublet; *t*, triplet; *q*, quadruplet. [b] Position with respect to the major substituent.

Germany). A 2.30 mL portion of reagent 1 (solvent and dilution reagent), 200 μL of reagent 2 (buffer solution), 25 μL of reagent 3 (photosensitizer), and 10 μL of standard (Trolox solution in reagent 1) or sample (essential oil in methanol) solution were mixed and measured. A light emission curve was recorded over 130 s, using inhibition as the parameter to evaluate antioxidant potential. The antioxidant capacity was then determined by using the integral under the curve and was expressed as millimoles per liter of Trolox used as standard to obtain a calibration curve (**Table 3**). A detailed description of the method is given elsewhere (*24*).

Tests of Antimicrobial Activity. Microorganisms and Culture Methods. Biological activities (antifungal and antibacterial activities) of *O. bofo* samples and *T. vulgaris* essential oils were determined for different classes of microorganisms. For antibacterial assays, Gram-positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Bacillus subtilis* ATCC 7003) and Gram-negative (*Escherichia coli* ATCC 4350, *Pseudomonas aeruginosa* ATCC 17934, and *Klebsiella oxytoca* ATCC 29516) bacterial strains were employed. Antifungal activity was assessed on the yeasts *Candida albicans* ATCC 48274, *Rhodotorula glutinis* ATCC 16740, *Schizosaccharomyces pombe*

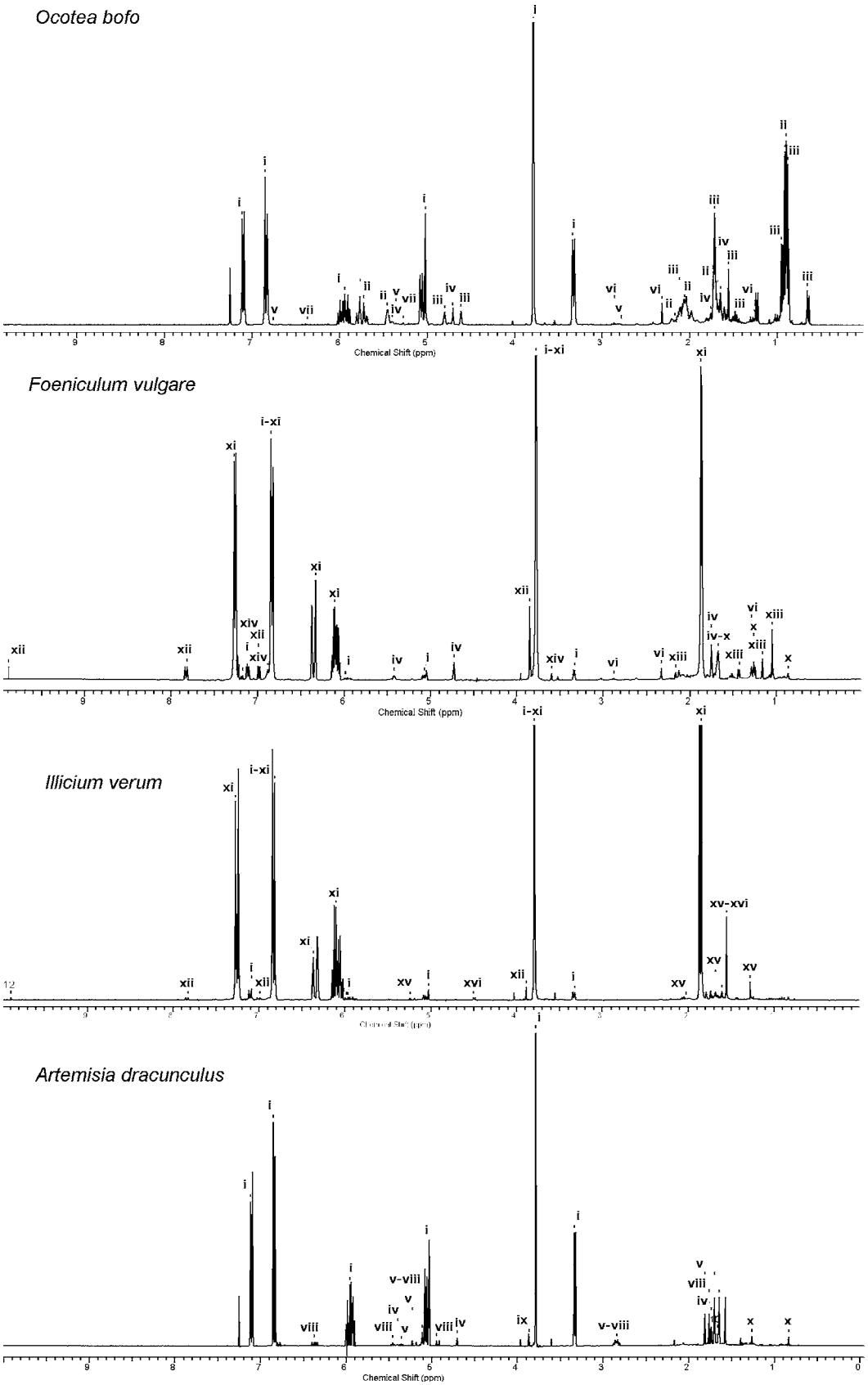


Figure 1. ^{1}H NMR spectrum of essential oils at 400 MHz.

ATCC 60232, *Saccharomyces cerevisiae* ATCC 2365, and *Yarrowia lypolitica* ATCC 16617. The culture media and conditions employed for ATCC strains were in accordance with American Type Culture Collections protocols (*26*).

Antimicrobial activity was determined by employing the standard disks diffusion technique (*27*). Mother cultures of each microorganism were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating from the mother cultures Petri dishes with proper sterile media with the aim of obtaining the microorganism concentrations of 10^5 and 10^6 CFU/mL for yeasts and bacteria, respectively. Aliquots of dimethyl sulfoxide (DMSO) were added to the essential oils to achieve solutions in the 0.01−0.6 mg/mL

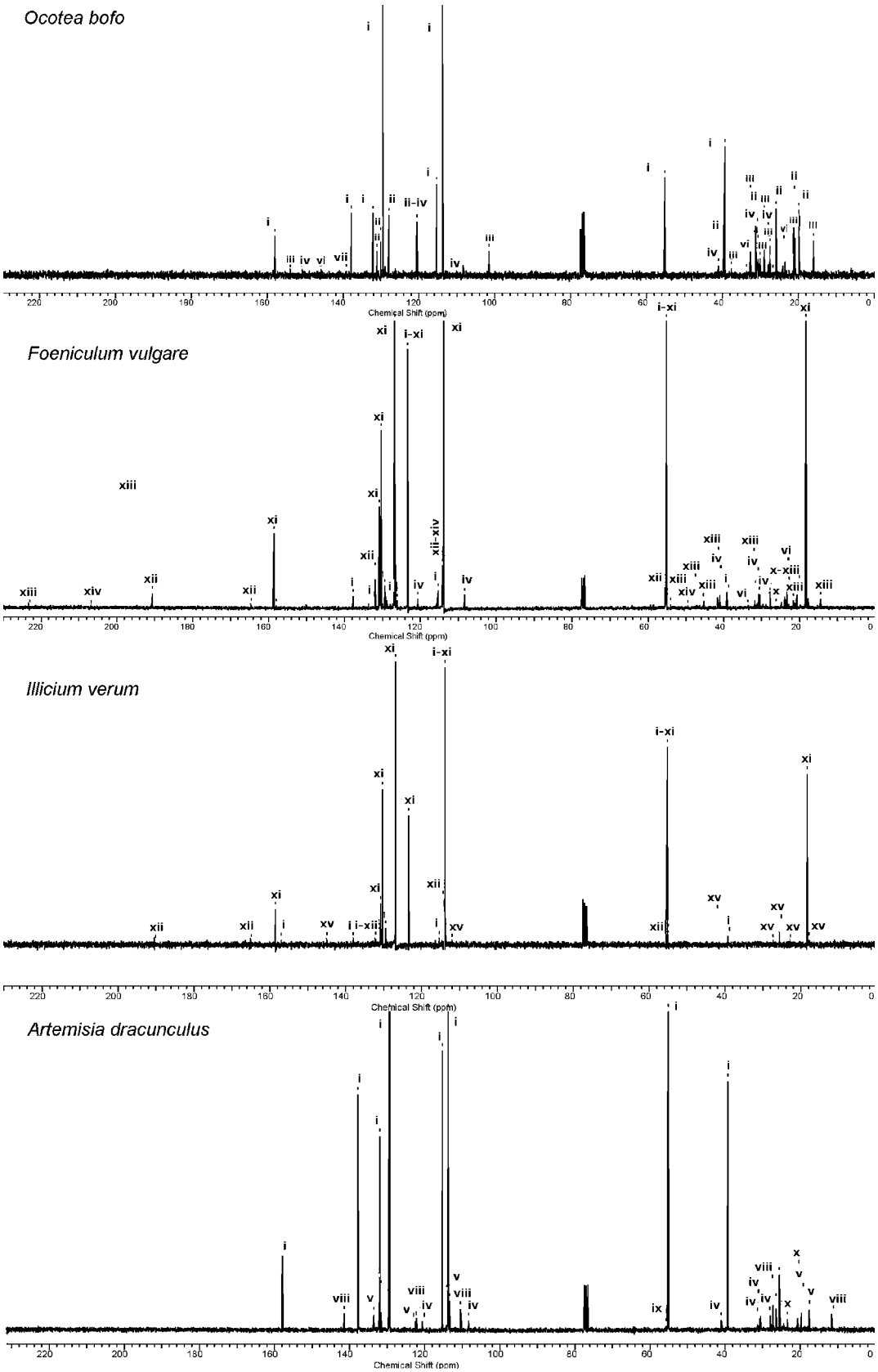


Figure 2. ^{13}C NMR spectrum of essential oils at 400 MHz.

range, and 10 μL of each dilution was deposited on a sterile paper disk (6 mm diameter, Difco). The lowest concentration of each essential oil showing a clear zone of inhibition was taken as the minimum inhibitory concentration (MIC) (*28*). Controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oil. GC-MS analysis of the oil led to the identification of 25 constituents (>95% of the total), listed in **Table 1**, along with their quantitative data and Kovat's indices. The major constituents

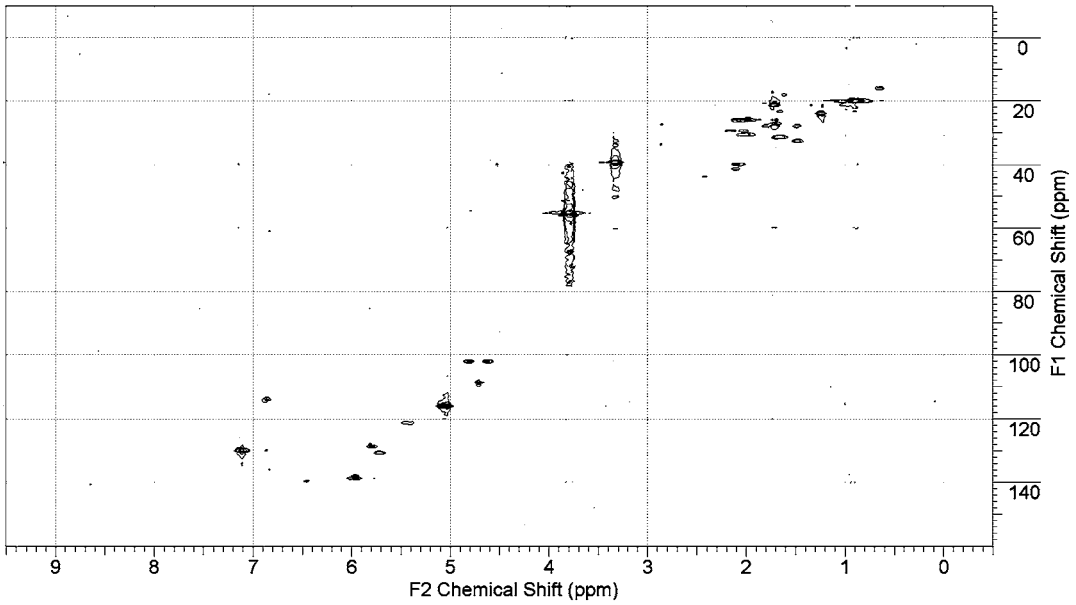


Figure 3. HSQC spectrum of *O. bofo* essential oil.

were mainly monoterpenes (45.3%); α-phellandrene (19.6%), sabinene (10.4%), limonene (5.0%), *p*-cymene (4.8%), *cis*-ocimene (1.8%), and *β*-myrcene (1.6%) were the most abundant, and their identification was confirmed by ^{13}C and ^{1}H NMR spectra. The sesquiterpene content was extremely low (<2.5%). The particular abundance of the phenylpropanoid estragole (48.7%) can be considered responsible for the Ecuadorian popular name of the plant, due to its anise-like perfume. If compared to *I. verum* and *F. vulgare*, *O. bofo* essential oil is distinguished by a higher abundance of estragole, α-phellandrene, and sabinene, whereas the main compound of star aniseed oil, anethole, is absent. *A. dracunculus* instead contains a large amount of estragole.

Such differences are clearly evidenced in both ^{1}H and ^{13}C NMR spectra, comparing the resonances in **Table 2** and **Figures 1** and **2**. Most significant zones in the ^{1}H NMR spectra are in the 6.80−7.90 ppm range (aromatic protons), the 4.50−6.80 ppm range (simple or α-conjugated double bonds), the 3.70−4.00 ppm range (methoxy groups of anisole), or the 2.00−3.70 ppm range (methylene groups in α of double bond). In the ^{13}C NMR spectru,. resonances from 190 to 230 ppm are characteristic for aldehydic and carbonylic carbons, resonances from 100 to 170 ppm for those of aromatic rings and double bonds, and resonances from 55 to 60 ppm for the methoxy group of anisole. In the ^{1}H NMR spectra (**Figure 1**), estragole (i) and *trans*-anethole (xi) showed signals overlapped at 3.79 and 6.84 ppm attributed to protons of methoxy group and to the nearest aromatic ring (H_2, H_6) (*29*). For anethole, the presence of propenyl instead of allyl caused a shift of H_3 and H_5 protons at lower fields, and thus this is quite distinctive in NMR fingerprinting of anethole/estragole oils. Moreover, in this regard, protons of the double bond exhibited resonances with characteristic multiplicity and coupling constants at 6.10 and 6.35 ppm, whereas those of estragole were at 5.04, 5.05, and 5.95 ppm. For anethole, the methyl group gave signals at 1.87 ppm (double doublet), whereas the methylene of estragole gave a signal at 3.33 ppm (doublet) (**Figure 1**). The HSQC and HMBC spectra confirmed data in the literature (*29*) about correlations of proton resonances to carbons (**Table 2**). In ^{13}C spectra carbons directly linked to protons that showed overlapping in ^{1}H NMR spectra exhibited the same chemical shifts in anethole and estragole, but other resonances were well separated, as are those of quaternary carbons (158.6 and 130.8 ppm for anethole and 157.9 and 132.0 ppm for estragole) (**Figure 2** and **Table 2**). For α-phellandrene (**ii**), some characteristic signals were evident: in the ^{1}H NMR spectrum resonances at 0.89 and 0.91 ppm (methyl groups) and particurarly at 5.46, 5.71, and 5.78 ppm (methyl group of double bonds); in the ^{13}C spectrum signals of unsaturated carbons (120.4, 127.9, 130.1, and 131.1 ppm). These assignments seem to be quite useful in the evaluation of the presence of this compound, the abundance of which could lower the quality of *O. bofo* essential oil and act as a marker in its distinction from *A. dracunculus* oil. According to HMBC correlations the multiplet at 5.46 ppm was attributed to H_1: in fact, it correlated to carbon at 120.4 ppm (HSQC) that showed connectivities (HMBC) with resonances at 1.74 and 5.78 ppm (**Figures 3** and **4**). The double doublet at 5.71 ppm was attributed to H_4 of the double bond that showed a large coupling constant with H_3 (correlation confirmed also by COSY experiment (**Figure 5**) and a small coupling constant with H_5 (**Table 2**) and correlated to resonance at 130.1 ppm (C_4): this carbon showed connectivities to 2.05 (H_5). The doublet of triplets at 5.78 ppm was attributed to H_3 and gave a large coupling constant to H_4 and small and equal coupling constants with H_1 and H_5 (so the double doublet of doublets degenerated to doublet of triplets). In the HSQC spectrum it was connected to resonance at 127.9 ppm. The C_2 quaternary carbon at 131.1 ppm was correlated to 5.71, 5.78, and 1.74 ppm (HMBC). The H_5, H_6, and H_7 protons were overlapped with other resonances, but it was possible to establish their connectivities and, consequently, assignments in COSY, HMBC, and HSQC spectra (**Table 2**). In the same way it was possible to attribute and correlate protons to carbons for sabinene (**iii**) (**Figures 1**−**5** and **Table 2**) (*29*). Most significant resonances were as follows: in the ^{1}H NMR spectrum, resonances at 0.66 ppm for protons of the cyclopropylic methylene group (H_6), at 0.87 and 0.94 ppm for those of methyl groups, at 1.49 ppm for a sextuplet of isopropylic methyne, and at 4.61 and 4.80 ppm for geminals of the double bond; in the ^{13}C spectrum, signals of unsaturated carbons (101.5 and 154.4 ppm) and of the methylene group of cyclopropyl (16.0 ppm).

It was also possible to identify some characteristic signals for limonene (**iv**), present also in *A. dracunculus* and *F. vulgare*), *cis*-*Z*-ocimene (**v**), *p*-cymene (**vi**), and myrcene (**vii**) (**Table 2**

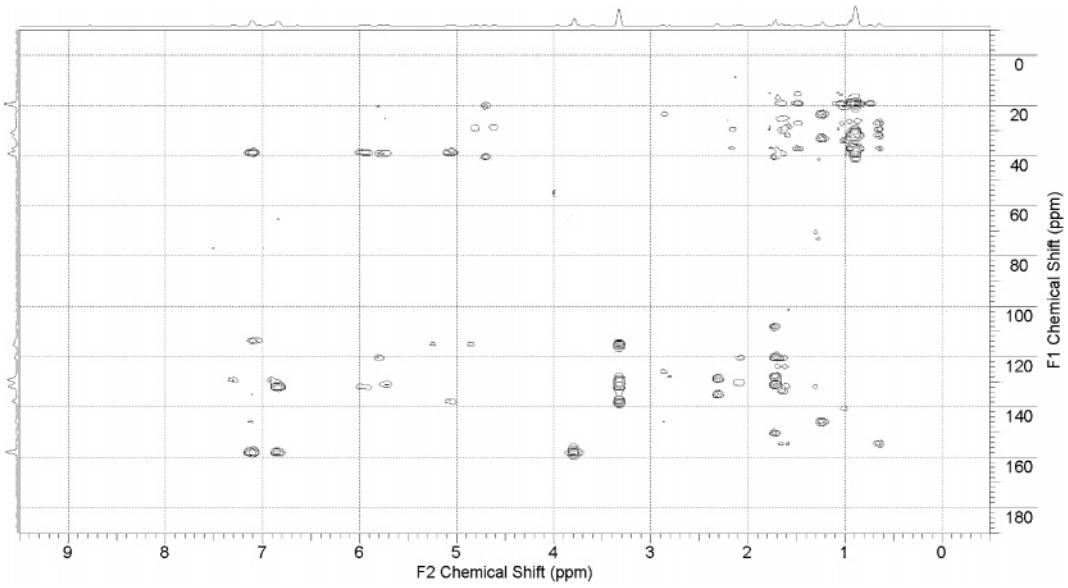


Figure 4. HMBC spectrum of *O. bofo* essential oil.

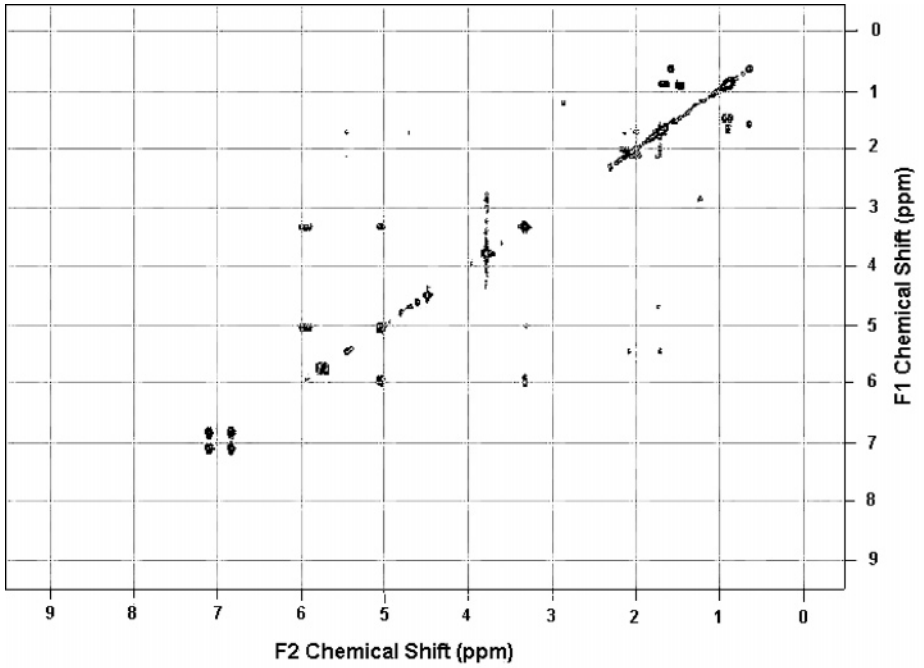


Figure 5. COSY spectrum of *O. bofo* essential oil.

and **Figures 1** and **2**). Moreover, in *A. dracunculus* were well distinguished resonances for the two isomers of ocimene (compounds **v** and **viii** in **Table 2** and **Figures 1** and **2**) and distinctive signals (methoxy groups) of methyleugenol from estragole. These differences could be extremely useful for a fingerprint discrimination of *O. bofo* and *A. dracunculus* essential oils. *F. vulgare* and *I. verum* showed typical resonances at low fields of *p*-anisaldehyde (**xii**), whereas only the first one exhibited signals for fenchone (**xiii**) and 4-methoxyphenylacetone (**xiv**) and the second for linalool (**xv**) and foeniculin (**xvi**).

Functional Properties of *O. bofo* Essential Oil. Estragole-rich essential oils are reputed for their antifungal properties (*30*); thus, we focused our attention on the activity against both pathogenic yeasts and bacterial strains (**Table 3**). Results obtained from bacterial strains evidenced a higher sensitivity of *E. coli*, *S. aureus*, and *B. subtilis*, whereas *P. aeruginosa* was not affected by the oil, which otherwise provided similar or slightly higher MICs than *T. vulgaris*. A higher efficacy was detected against yeasts, with the sole exception of *S. cerevisiae*, which showed a higher MIC. This different behavior may, in part, be associated with the abundance of estragole, which possesses an elective specificity for fungal strains rather than for bacteria (*30*).

With the aim to provide preliminary information on the functional properties of *O. bofo* essential oil, its in vitro antioxidant activity was assessed with three different tests: the DPPH test, the β-carotene bleaching test, and the luminol PCL assay, which allow to be followed both the primary and secondary steps of oxidation (*31*) and the lipid soluble antioxidant capacity. This multiple approach permits the antioxidant effectiveness of an essential oil to be more carefully defined, as it is almost impossible to express the antioxidant activity as an absolute value universally recognizable (*32*). The results were compared to the activities of synthetic antioxidants (BHA, Trolox, and α-tocopherol) and to *T. vulgaris* commercial essential oil (thymol chemotype), taken as a natural reference.

Table 3. Antimicrobial Activity Expressed as Minimum Inhibitory Concentration (MIC) of *O. bofo* Essential Oil Compared with That of Commercial *T. vulgaris* Essential Oil Taken as Positive Control

	MIC[a] (mg/mL)		
	O. bofo	*T. vulgaris*	antibiotic[b]
bacteria			
Staphylococcus aureus subsp. *aureus* ATCC 29213	0.16	0.11	0.1×10^{-3}
Bacillus subtilis ATCC 7003	0.16	0.11	0.1×10^{-3}
Enterococcus faecalis ATCC 29212	0.32	0.11	0.1
Pseudomonas aeruginosa ATCC 17934	NA[c]	0.18	0.5
Klebsiella oxytoca ATCC 29516	0.32	0.4	0.4
Escherichia coli ATCC 4350	0.16	0.06	0.06
yeasts			
Saccharomyces cerevisiae ATCC 2365	0.52	0.03	0.15×10^{-3}
Rhodotorula glutinis ATCC 16740	0.13	0.09	0.18×10^{-3}
Schizosaccharomyces pombe ATCC 16740	0.07	0.09	0.18×10^{-3}
Yarrowia lypolytica ATCC 16617	0.10	0.06	0.20×10^{-3}
Candida albicans ATCC 48274	0.15	0.14	0.25×10^{-3}

[a] The MIC was considered as the lowest concentration of each essential oil showing a clear zone of inhibition. [b] Ampicillin and fluconazole were employed to test the sensitivity of bacteria and yeasts, respectively. [c] Not active. MIC was >0.75 mg/mL.

Table 4. Antioxidant Activity of *O. bofo* Essential Oil Performed by DPPH and β-Carotene Bleaching Assays[a]

	inhibition % ± SD		PCL
sample	DPPH	β-carotene bleaching test	(mmol of Trolox/L)
O. bofo essential oil	64.23 ± 0.03	75.82 ± 0.04	3.14 ± 0.02
T. vulgaris essential oil	75.64 ± 0.04	90.94 ± 0.05	0.34 ± 0.06
BHA	84.35 ± 0.04	86.74 ± 0.04	
α-tocopherol			4.28 ± 0.5
Trolox	94.44 ± 0.05	84.60 ± 0.04	3.94 ± 0.6

[a] The results achieved were compared to those of synthetic references butylated hydroxyanisole (BHA) and Trolox and to that of *T. vulgaris* essential oil taken as natural reference.

In the DPPH test, *O. bofo* essential oil revealed a remarkable scavenging effect. In fact, the antiradical activity expressed as DPPH inhibition percentage was higher than that of Trolox and slightly lower than that of thyme essential oil (**Table 4**). This behavior was confirmed in the β-carotene bleaching test, where the protective action of the oil against linoleic acid peroxidation was comparable with the ones provided by both controls.

The PCL assay was chosen for its particular suitability to determine the radical scavenging activity of lipid-soluble antioxidants such are essential oils (*25*).

Results obtained for *O. bofo* oil were consistently better than the natural reference oil and almost equal to those provided by both α-tocopherol and Trolox. These data are of particular significance because the results of this assay easily correlate with the therapeutical, nutraceutical, and cosmeceutical potential of a given antioxidant, the capability to quench $O_2^{\bullet -}$ being useful to describe the related capacity to counteract ROS induced damages to the body.

The *Ocotea* genus hosts important timber trees, which are subject to be felled during the deforestation of the rainforest habitat. Therefore, their eventual exploitation as sources of profitable and sustainable nontimber products deserves particular attention. The chemical composition of the essential oil obtained from *O. bofo* floral calyces was found to be similar to that of *A. dracunculus*, suggesting that this Amazonian tree could be considered to be a source of an estragon substitute. Besides its aromatic properties, *O. bofo* essential oil provided also interesting properties under a functional perspective. In fact, it combines fair antioxidant and antiradical effects with a fair antimicrobial action, covering both the inhibition of food-related yeasts and contaminating bacteria. In view of its use as a possible adulterant of estragole- or anethole-rich oils, an exhaustive NMR fingerprinting comparison has been conducted with *F. vulgare*, *I. verum*, and *A. dracunculus* oils. The achieved data suggest that this nonchromatographic approach may be suitable for the identification, quality control, or fraud detection of essential oils with a similar phenylpropanoid pattern, providing their good and fast discrimination.

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Received for review February 24, 2006. Revised manuscript received July 5, 2006. Accepted July 9, 2006.

JF0605493