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Composition and Antioxidant Activity of the Essential Oils of *Xylopia aethiopica* (Dun) A. Rich. (Annonaceae) Leaves, Stem Bark, Root Bark, and Fresh and Dried Fruits, Growing in Ghana

ANASTASIA KARIOTI,[†] DIMITRA HADJIPAVLOU-LITINA,[‡] MERLIN L. K. MENSAH,[§] THEOPHILUS C. FLEISCHER,[§] AND HELEN SKALTSA*,[†]

Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Panepistimiopolis, Zografou 15771, Greece, Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece, and Department of Pharmacognosy, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

The chemical composition of the essential oils obtained from the leaves, the barks of the stem and the root, as well as from the fresh and dried fruits of *Xylopia aethiopica*, growing in Ghana, was investigated by gas chromatography/mass spectrometry analyses. Kováts indices, mass spectra, and standard compounds were used to identify a total of 93 individual compounds. The monoterpene hydrocarbons formed the main portion in all studied samples. β-Pinene was predominant in all cases, while trans-*m*-mentha-1(7),8-diene was the main compound in the essential oils of the leaves and the barks of roots and stems. Their potential antioxidant activity was also investigated and found to be significant in scavenging superoxide anion radical.

KEYWORDS: ***Xylopia aethiopica*; Ghana; volatile constituents; leaves; fruits; stem bark; root bark**

INTRODUCTION

The genus *Xylopia* (Annonaceae) comprises many species, which occur in the tropics, especially Africa (*1*). The West African pepper tree, *Xylopia aethiopica* (Dun) A. Rich, is a tree that grows wild in Ghana. Its fruit is a black berry and contains 4−9 peppery seeds (*1*). Both fruits and seeds are used as condiments or mixed spices (*1*). The average consumption level varies between 1 and 10 g per day. Normally, the fruit containing the seeds is pounded and used in cooked foods or in the spicing of beverages. As a component of herbal medicines, it is locally used as a carminative, stimulant, and additive to other remedies for the treatment of skin infections, as digestive, appetizer, and antiemetic agents, and for the management of cough and fever (*2*).

Although many studies of the fruits of *X. aethiopica* have been performed (*3*−*6*), no detailed research has been reported on the essential oil of the other parts of the plant. Previous studies are focused on the chemical composition of essential oils, obtained from dried fruits of *X. aethiopica* from different regions of Africa using gas chromatography/mass spectrometry (GC/MS) analysis (*3*−*6*). The results of these studies show a variety of monoterpenes and sesquiterpenes in the content of the oils. β-Pinene seems to be ubipresent, in some cases with a remarkably high proportion (*3*, *6*), while 1,8-cineole is also detected in all cases but in a smaller percentage. Sabinene was the main constituent of the dried fruits from Benin (*5*), while the fruits collected in Egypt were rich in terpinen-4-ol and α-terpineol (*3*).

This work aims to provide information on the composition and antioxidant activity of the essential oils of leaves, barks of stems and roots, and fresh and dried fruits of *X. aethiopica* growing wild in Ghana. The property of many herbs and spices to conserve food has been associated with their antibacterial and antioxidant activities, which are attributed to their phenolic content. Spices are considered to be important natural antioxidants (*7*−*9*) and hold promise as an alternative solution in the food industry as they seem to lack the adverse effects of currently used synthetic antioxidants. In this framework, the antioxidant activity of the essential oils of *X. aethiopica* was estimated.

MATERIALS AND METHODS

Plant Material. The leaves, the barks of the stem and the root, and the fresh and dried fruits of *X. aethiopica* were collected in the morning (temperature range, 27−31 °C) from the same plant growing in the physique garden of the Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology (KNUST) campus, in January 2003, and identified by comparing them with herbarium specimens at the Forestry Department, Kumasi. The plant was cultivated from the seedlings obtained from the botanical garden of the University six years ago. The fruits were of different sizes (0.5−6.0 mm). Some of the fresh fruits were air-dried (in shade, at temperatures ranging between 27 and

* To whom correspondence should be addressed. Tel: +30 210 7274593. Fax: +30 210 8613965. E-mail: skaltsa@pharm.uoa.gr.
[†] University of Athens.
[‡] Aristotle University of Thessaloniki.
[§] Kwame Nkrumah University of Science and Technology.

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31 °C) for 14 days prior to distillation of the oil. A voucher specimen (no. FP/PH/XE10203/TCF) has been deposited at the Pharmacognosy Department Herbarium, KNUST.

Isolation of the Essential Oils. The leaves (1.30 kg), stem bark (1.00 kg), root bark (0.65 kg), and the fresh and dried fruits (0.60 and 0.25 kg, respectively) were weighed, cut into pieces, and packed for extraction. The essential oil obtained after 3 h of hydrodistillation using the BP apparatus (*10*) from each sample was measured (mL) and collected in a small beaker to which a small amount of anhydrous sodium sulfate was added to absorb water (*11*) and then was kept in a stopped vial at −15 °C for further analysis. The yield is the mean of two replicates. The oils were diluted in capillary GC grade *n*-pentane (1:20), and 1 μL was subsequently analyzed by GC and GC/MS. The composition of the volatiles was resolved utilizing flame ionization detection (FID)-GC.

GC. Analysis was carried out on a Perkin-Elmer 8500 gas chromatograph with FID, fitted with a fused silica DB-5 MS capillary column [30 m × 0.25 mm (i.d.); film thickness, 0.25 μm]. The column was temperature programmed from 60 to 250 °C at a rate of 3 °C/min. The injector and detector temperatures were programmed at 230 and 280 °C, respectively.

GC/MS. The essential oils were analyzed using capillary GC/MS system operating in the EI mode. GC/MS analyses were performed on a Hewlett-Packard 5973-6890 system operating in EI mode (70 eV) equipped with a split/splitless injector (220 °C), a split ratio 1/10, using three different columns: a fused silica HP-5 MS capillary column [30 m × 0.25 mm (i.d.); film thickness, 0.25 μm], a HP-Innowax capillary column [30 m × 0.25 mm (i.d.); film thickness, 0.50 μm], and a chiral Cydex B capillary column [50 m × 0.22 mm (i.d.); film thickness, 0.25 μm]. The temperature program for the HP-5 MS column was from 60 (5 min) to 280 °C at a rate of 4 °C/min; for the HP-Innowax column, it was from 60 to 260 °C at a rate of 3 °C/min; and for the Cydex B column, it was from 50 to 130 °C (2 min) at a rate of 2 °C/min and from 130 to 250 °C at a rate of 4 °C/min. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The injection volume of each sample was 2 μL.

Retention indices for all compounds were determined according to the Van der Dool approach (*12*), using *n*-alkanes as standards. The identification of the components was based on comparison of their mass spectra with those of Wiley and NBS Libraries (*13*) and those described by Adams (*14*), as well as by comparison of their retention indices with literature data (*14*, *15*). In many cases, the essential oils were subjected to cochromatography with authentic compounds (Aldrich, Fluka, Sigma). The recognition of the optical isomers was made by comparison with authentic samples and according to reported elution order for the particular column (*16*−*18*). The optical rotation values were determined at 25 °C at 589 nm in $CHCl_3$ (Uvasol). Polarimeter: Perkin-Elmer 341. A Perkin-Elmer Lambda 20 UV−vis spectrophotometer was used for the radical scavenging activity experiments.

In Vitro Assays. For the tested essential oils, stock solutions were prepared in absolute ethanol. The g/1 mL ethanol in each case is given in **Table 2**. A Perkin-Elmer Lambda 20 UV−vis spectrophotometer was used for the radical scavenging activity experiments.

Competition of the Tested Compounds with Dimethyl Sulfoxide (DMSO) for Hydroxyl Radicals. The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system were detected according to Nash (*19*), by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained ethylenediaminetetraacetic acid (0.1 mM), Fe^{3+} (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compound, and 100 and 150 μL of ascorbic acid (10 mM in phosphate buffer was added at the end in order to have the reaction started). After 30 min of incubation (37° C), the reaction was stopped with CCl_3COOH (17% w/v) and the formaldehyde formed was detected spectrophotometrically at 412 nm (**Table 2**). Trolox used as an appropriate standard gave 88.2% in 0.1 mM.

Interaction of the Tested Compounds with 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Stable Free Radical. Twenty microliters from the stock solution of each sample was diluted in absolute ethanol to a final volume of 1 mL and then added to 1 mL of DPPH (0.1 mM, in absolute ethanol). The reaction mixture was then vigorously stirred for 10 s and allowed at room temperature for 20 and 60 min. As control, a solution with the same chemicals, except for the samples, was used (*20*). Ethanol was used to zero the spectrophotometer. The optical density (OD) of the solution was measured, and the percent reduction was estimated with the following equation at 517 nm. The ODs of the samples without the presence of DPPH were recorded and subtracted from the corresponding OD with DPPH.

$$\%\ \text{reduction} = \frac{\text{control OD (mean)} - \text{sample OD (mean)}}{\text{control OD (mean)}} \times 100$$

Acetylsalicylic acid used as an appropriate standard gave 80.6% in 0.1 mM (**Table 2**).

Scavenging Activity of Superoxide Anion Radical. The superoxide anion was generated by the xanthine−xanthine oxidase system and measured by the nitroblue tetrazolium (NBT) method (*21*). A reaction mixture in phosphate buffer, pH 7.4 (0.1 mol/L), containing 350 μL of xanthine, 150 μL of NBT, and 100 μL of each sample was prepared. Forty microliters (50 units/2.6 mL) of xanthine oxidase was subsequently added. After the mixture was incubated for 10 min at room temperature, the absorbance was recorded at 560 nm (**Table 2**). Each of the above experiments was performed at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oils. The yields (v/w) of the essential oils are shown in **Table 1**. As it is shown, the essential oils from all of the plants are complex mixtures of compounds with contributions of main products never exceeding 30.5% of the total. Among them, the monoterpene hydrocarbons formed the main portion in all studied samples, while leaf and fresh and dried fruit essential oils had a high content in germacrene D (24.5, 19.4, and 25.1%, respectively). Generally, all samples were characterized by the absence of phenylpropanoids, fatty acids, aliphatic esters, and diterpenoids with the exception of kaur-16-ene, present in trace amounts or in very low amounts in most investigated samples. The main constituent of both root and stem bark essential oils is *trans*-*m*-mentha-1(7),8-diene (30.4 and 30.7%, respectively). All samples contain remarkable amounts of α-/β-pinene. Germacrene D is also present in high quantities in all samples with the exception of the root bark essential oil (relatively lower amount, 1.0 vs 8.8−25.1%); the latter is characterized by the presence of the oxygenated sesquiterpene β-copaen-4-α-ol (13.3%), which is totally absent in the other samples. It is noteworthy that germacrene D has not been previously detected (*5*) or it has been reported to be in traces (*6*).

Previous investigations on the essential oils of the fruits of *X. aethiopica* from various areas revealed that they mainly consist of mono- and sesquiterpenes, among which α-and β-pinene, myrcene, *p*-cymene, limonene, linalool, terpinen-4-ol, α-terpineol, and 1,8-cineole are the most predominant (*3*, *22*−*25*). Other identified compounds were β-phellandrene, (*E*)-β-ocimene, and bisabolene (*25*). Cumine aldehyde has been identified in the fruit essential oil of *X. aethiopica* from Egypt (*3*) and from Nigeria (*26*) in contrast to our samples, where it is practically absent. Sabinene has been found in the fruit essential oil from Nigeria (*25*) and from the Republic of Benin (36.0%) (*27*), while in our samples it was detected only in traces.

Elemol has been identified in the essential oils of the fruits and the leaves of *X. aethiopica* growing wild in the Republic of Benin (*27*) and in Cameroon (*28*, *29*). Concerning our samples, elemol was identified in relatively high amounts (4.8%) only in the essential oil of the leaves.

The use of a chiral column allowed the determination of enantiomers in several main compounds. In most cases, only one isomer is present in the essential oil, the other being absent

Table 1. Qualitative and Quantitative Composition (% v/v) of Volatile Compounds in Leaves, Root Bark, Stem Bark, and Fresh and Dried Fruits of *Xylopia aethiopica* from Ghana[a]

	RI[b]								RI[b]							
	1	2	leaves	root bark	stem bark	fresh fruit	dried fruit	identification[c]	1	2	leaves	root bark	stem bark	fresh fruit	dried fruit	identification[c]
tricyclene[c]	925			0.1				d	α-cubebene	1350	1342	0.2			0.3	0.4	c-d [a]
(–)-α-pinene	938		5.3	1.5	7.3	6.4	5.0	c-d [a]	α-terpinyl-acetate	1354			0.4	1.7			c-d [f]
(+)-α-pinene			2.0	2.0	2.4	7.9	3.0	c-d [a]	α-ylangene[c]	1371		0.1				0.1	d
camphene	948		0.2	5.6			0.1	c-d [a]	α-copaene	1377	1373	1.2	0.1	0.9	2.7	3.8	c-d [f]
sabinene	975	1017		0.1				c-d [f]	β-cubebene[c]	1388	1423		0.1		0.4		d
(–)-β-pinene	981	1008	17.3	1.2	5.8	25.5	21.6	c-d [a]	β-elemene[c]	1389	1470	1.4			0.6	1.1	d
(+)-β-pinene				7.1				c-d [a]	geranyl acetate	1379			0.1				c-d [a]
(+)-δ-2-carene	993						4.5	c-d [a]	β-patchoulene[c]	1388			1.3				d
trans-m-mentha-1(7),8-diene[c]	1008	1055	3.0	30.4	30.7	5.8		d	cyperene[c]	1401	1405	0.1	2.7	7.6	1.1	1.7	d
α-phellandrene	1001	1160	0.6		2.5	1.9	1.0	c-d [f]	(–)-α-gurjunene	1409				4.5	0.9	1.0	c-d [f]
α-terpinene	1017		0.1	0.1	0.2	0.5	0.3	c-d [a]	longifolene	1407			0.5	0.6			c-d [a]
p-cymene	1024	1166		0.3	0.1			c-d [a]	*trans*-caryophyllene[c]	1418	1475	5.2	0.8	1.0	0.2		d
1,8-cineole	1030	1110		1.8			7.4	c-d [a]	α-guaiaene[c]	1426				0.1			d
(–)-limonene	1031	1099		0.8			0.8	c-d [a]	γ-elemene[c]	1434	1515	0.5			0.4	0.4	d
cis-ocimene	1033		2.2					c-d [f]	β-gurjunene[c]	1439	1666		0.6			1.0	d
β-phellandrene[c]	1036		1.6		2.8	9.7		d	α-humulene	1454	1560	1.1	0.1	0.9	0.9		c-d [s]
trans-β-ocimene[c]	1050	1147	0.5		0.1	0.1	tr	d	α-amorphene[c]	1485	1575				0.3	1.6	d
γ-terpinene	1062		0.1	0.2	0.5	0.7	0.6	c-d [a]	germacrene D[c]	1486	1612	24.5	1.0	8.8	19.4	25.1	d
cis-sabinene hydrate[c]	1067	1352			0.1	0.1	0.1	d	β-selinene[c]	1488	1618		0.4	0.5			d
α-terpinolene	1089		0.1	0.2	0.4	0.3	0.2	c-d [f]	α-selinene[c]	1494	1623		0.6	1.2			d
(–)-linalool	1100	1424	0.2		0.2	0.4	0.3	c-d [a]	valencene[c]	1496		1.5	0.3	0.4			d
endo-fenchol	1117				0.1			c-d [a]	*cis*-cadina-1,4-diene[c]	1497		0.4			0.2	0.3	d
cis-p-menth-2-en-1-ol[c]	1119				0.2			d	γ-cadinene[c]	1513		0.4	1.0	0.6		0.5	d
α-camphonellal	1125				0.1			d	δ-cadinene	1523	1655	1.2	0.2	1.6	0.8	1.8	c-d [f]
allo-ocimene	1132		0.1			tr		d	*trans*-cadina1(2),4-diene[c]	1535	1973	0.1		0.1	0.2		d
1-terpineol	1134	1440		tr	0.5	0.1	0.3	c-d [f]	α-cadinene[c]	1531	1785	0.1		0.1	0.1		d
trans-pinocarveol	1139	1447	0.1					c-d [f]	*trans*-calamenene[c]	1532	1738			2.2			d
cis-verbenol[c]	1141				tr			d	elemol	1547	1988	4.8			0.2	0.2	c-d [f]
camphor	1146			tr				c-d [a]	germacrene B[c]	1561	1734				0.4	0.7	d
camphene hydrate[c]	1147			0.2	0.2			d	*endo*-1-bourbonanone[c]	1564	2052	0.1			0.1		d
pinocarvone[c]	1157	1447	tr		0.2	0.1	0.1	d	β-copaen-4-α-ol[c]	1570			13.3				d
borneol	1166	1602		0.1	0.2		-	c-d [a]	(+)-spatulenol[c]	1576	2030			tr		0.1	d
δ-terpineol[c]	1162					0.2	-	d	caryophyllene oxide	1583	1892	0.1		0.1			c-d [a]
terpinen-4-ol	1178	1481	0.3	0.4	1.3	1.7	1.7	c-d [f]	guaiol[c]	1599	1997	3.6			0.1	0.1	d
α-terpineol	1185	1598	0.4	0.4	2.2	1.6	2.2	c-d [a]	T-cadinol[c]	1640	2165	0.4	2.3	0.1	0.1		d
myrtenal	1189	1509	0.2				0.1	c-d [a]	T-muurolol[c]	1641	2145		0.4				d
verbenone	1201						0.1	c-d [a]	torreyol[c]	1643	2180			0.1			d
myrtenol	1203	1697			0.2		0.1	c-d [a]	β-eudesmol[c]	1647				0.1			d
cis-piperitol[c]	1211			tr	tr	0.1		d	α-eudesmol[c]	1650		0.9					d
trans-carveol	1217				tr		0.2	c-d [a]	α-cadinol[c]	1650	2225		0.4	1.0			d
nerol	1228	1820			0.1			c-d [a]	bulnesol[c]	1672		0.8		0.1	0.1		d
cumin aldehyde	1240				tr			c-d [f]	juniper camphor[c]	1690				tr			d
piperitone[c]	1247		tr		tr			d	benzyl benzoate[c]	1764				tr			d
thymyl methyl acetate[c]	1235			0.1				d	*ent*-pimara-8(14),15-diene[c]	1939	2133		0.1	0.1			d
carvacrol methyl ether[c]	1244			tr				d	manoyl oxide[c]	2001							d
(–) bornyl-acetate	1280	1460		5.3	0.5		tr	c-d [a]	kaur-16-ene[c]	2032		0.1		tr	0.1	0.1	d
δ-elemene[c]	1332	1356	4.6			5.7	6.9	d									

Grouped Components

monoterpene hydrocarbons	33.0	49.6	52.8	58.8	37.1	oxygenated sesquiterpenes	10.7	16.4	1.5	0.6	0.4
oxygenated monoterpenes	1.3	8.7	7.8	4.3	12.6	diterpenoids	0.1	0.1	0.1	0.1	0.1
sesquiterpene hydrocarbons	42.6	9.8	31.1	34.6	46.4	total identified (%)	87.7	84.6	93.3	98.4	96.6

[a] Components listed in order of elution from a HP 5MS column. [b] RI, Kováts indices calculated against C_9–C_{24} *n*-alkanes on the HP 5MS column (1) and HP Innowax (2) capillary columns, respectively. Concentrations between 0.01 and 0.05 are marked as tr (traces). [c] Identification: c, comparison with authentic compounds; d, comparison of mass spectra with MS libraries. [a], [f], [s]: Reference compounds were provided from Aldrich, Fluka, and Sigma, respectively. [d] Tentatively identified.

or present in trace amounts. The recognition of the optical isomers was made by comparison with authentic samples and according to reported elution order for the particular column (*16*−*18*). The yields and the optical rotation values are given in **Table 2**.

The potential of the antioxidant activity is shown in **Table 3**. The antioxidant activity of the essential oils of the *X. aethiopica* has been evaluated in a series of in vitro tests. In view of the differences among the test systems avaiIable, the results of a single assay can give only a suggestion on the protective potential of phytochemicals. Among the plethora of methods used for the evaluation of the antioxidant activity, the DPPH test is very useful in the micromolar range demanding minutes to hours for both lipophilic and hydrophilic samples. In literature, many studies concerning the antioxidant activity of essential oils tested with DPPH have been reported (*30*−

Table 2. Yield and Optical Rotation of the Studied Essential Oils

	leaves	root bark	stem bark	fresh fruit	dried fruit
yield (%, v/w)	0.46	0.92	0.80	3.67	3.33
$[\alpha]_D^{20}$	−9.3 ($CHCl_3$, c, 0.43)	+2.1 ($CHCl_3$, c, 0.43)	−11.6 ($CHCl_3$, c, 0.42)	+3.6 ($CHCl_3$, c, 0.47)	+5.0 ($CHCl_3$, c, 0.42)

Table 3. Effects of the Examined Oils and Reference Drugs on the Mediated Oxidation of Dimethyl Sulfoxide (33mM) $HO^\bullet$ % in Vitro, % Interaction of the Examined Oils and Reference Drugs with the Stable Free Radical DPPH, and % Scavenging Activity of Superoxide Anion Radical

	[a]$HO^\bullet$ % compt.	% interaction[b] DPPH 20 min	% interaction DPPH 60 min	% scavenging[c] $O_2^{-\bullet}$
leaf oil (0.048 g/ 1 mL EtOH)	no	43.8	75.9	86.8
root bark oil (0.033 g/ 1 mL EtOH)	no	36.5	43.2	68.4
stem bark oil (0.037 g/ 1 mL EtOH)	no	32.4	40.3	73.9
fresh fruit oil (0.045 g/ 1 mL EtOH)	8.5	85.6	85.8	66.5
dried fruit oil (0.042 g/ 1 mL EtOH)	no	21.2	54.8	57.2

[a] Trolox as standard 96.4%. [b] Acetylsalicylic acid as a standard 80.6%. [c] Caffeic acid as a standard 70%. no, no result under the reported experimental conditions. Perkin-Elmer Lambda20 UV–vis spectrophotometer.

33). All tested samples were found to interact with the stable free radical DPPH in a time-dependent manner. In the presence of an antioxidant, which can donate an electron to DPPH, the purple color is typical of the free DPPH radical decay; a change spectrophotometrically (517 nm) followed. This interaction indicates their radical scavenging ability in an iron-free system. In cases where the structure of the electron donor is not known (e.g., plant extract), this method can afford data on the reduction potential of the sample and, hence, can be helpful in comparing the reduction potential of unknown materials. The dried fruit essential oil produced approximately 21.2% interaction, whereas the essential oils of the root bark and stem barks were found to interact satisfactorily (32.4 and 36.5%, respectively). The essential oil of the leaves was found to possess a 43.8% interaction. The essential oil of the fresh fruits showed the highest interaction (85.6%). In general, this interaction expresses the reducing activity of the tested samples and indicates their ability to scavenge free radicals.

The competition of the tested samples with DMSO for $HO^\bullet$ generated by the Fe^{3+}/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. In the present experiments, no inhibition was found.

Superoxide anion radical generated by the xanthine−xanthine oxidase system was spectrophotometrically measured by monitoring its ability to reduce nitroblue tetrazolium NBT to formazan. This assay was adopted to assess the ability of antioxidants to react with $O_2^{\bullet-}$. All tested samples (100 μL) at a final volume of 700 μL, showed a significant ability to scavenge superoxide anion radical (57.2−86.8%). The essential oil of the leaves showed the highest ability. Because of solubility problems, it was not possible to determine the scavenging activity of the samples in higher concentrations. On the contrary, lower concentrations did not show any significant result (data not shown).

Xanthine oxidase is a key enzyme that catalyzes the oxidation of hypoxanthine to xanthine. In the presence of molecular oxygen as an electron acceptor, xanthine generates uric acid, superoxide anions, and hydrogen peroxide. These reactions are as follows (*34*):

$$\text{hypoxanthine} + O_2 + H_2O \rightarrow \text{xanthine} + H_2O_2$$

$$\text{xanthine} + 2O_2 + H_2O \rightarrow \text{uric acid} + 2O_2^{\bullet-} + 2H^+$$

$$\text{xanthine} + O_2 + H_2O \rightarrow \text{uric acid} + H_2O_2$$

XOD-derived superoxide anions have been linked to post-ischemic tissue injury and edema, as well as to changes in vascular permeability. The inhibition of this enzyme is therefore useful in the treatment of several diseases (*35*). Several compounds present in plants are reported to be inhibitors of XOD (*35*) and to possess antioxidant properties.

The presence of (a) germacrene-D, a 10-membered ring system and the three double bonds acting as electron rich centers; (b) α- and β-pinenes; and (c) *trans-m*-mentha-1(7),8-diene is implicated to the antioxidative free radical scavenging activity. However, it is possible that minor compounds might play a significant role in the antioxidant activity (*36*, *37*) and synergistic effects should be taken into account.

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