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Evaluation of Antioxidant Activity of Vetiver (*Vetiveria zizanioides* L.) Oil and Identification of Its Antioxidant Constituents

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Antioxidant capacities of vetiver (*Vetiveria zizanioides*) oil were evaluated by two different in vitro assays: the DPPH• free radical scavenging assay and the Fe^{2+}-metal chelating assay. Results showed that the vetiver oil (VO) possessed a strong free radical scavenging activity when compared to standard antioxidants such as butylated hydroxytoluene (BHT) and α-tocopherol. However, its metal chelating capacity was relatively weak. VO (10 μL/mL) dissolved in methanol exhibited ∼93% free radical scavenging activity in the DPPH• assay and ∼34% Fe^{2+} chelating activity in the metal chelating assay. By contrast, 10 mM BHT and 0.1 mM α-tocopherol exhibited 93 and 89% free radical scavenging activities in the DPPH• assay, respectively, and 1 mM EDTA exhibited ∼97% activity in the metal chelating assay. Among the complex constituents in the crude VO, β-vetivenene, β-vetivone, and α-vetivone, which had shown strong antioxidant activities, were isolated and identified using various chromatographic techniques including silica gel open column chromatography, silica HPLC, and GC-MS. These results show that VO and some of its inherent components can be potential alternative natural antioxidants.

KEYWORDS: Vetiver oil; *Vetiveria zizanioides*; antioxidant activity; DPPH; terpenoids; vetivone

INTRODUCTION

Recently, essential oils extracted from various herbs and spices have been a subject of intensive research partially due to the continuous discoveries of their multifunctional properties other than their classical roles as food additives and/or fragrances. For example, antibacterial, antifungal, antioxidant, and anti-inflammatory activities of many essential oils have been investigated and confirmed (*1*−*11*). In recent years, the pharmacological action of essential oils from various aromatic and medicinal plants has been of great interest to both academia and pharmaceutical companies (*12*−*16*). In turn, so much investigation and new findings have significantly prompted and expanded novel applications of essential oils. In light of their novel functionalities and unique flavors, essential oils have now been widely used as natural insecticides, cosmeceuticals, and aromatherapeutic agents. Vetiver (*Vetiveria zizanioides* L.) is one of the most important raw materials in perfumery (*17*, *18*). Its root is usually steam distilled to obtain the vetiver oil. Except for termiticidal (*19*, *20*) and antimicrobial activities (*4*), the oil has not been studied intensively for its other biological functionalities, probably because of its complex constituents. VO is composed of more than 170 compounds that are mainly sesquiterpenes and their derivatives (*21*−*28*). Among the odorous components in vetiver oils from different sources, khusimol, β-vetivone, α-vetivone, etc., are the major constituents, and their presence is often considered as the fingerprint of the oil (*29*). Nevertheless, to our knowledge, the antioxidant activity of VO and its components has not been reported so far.

Many studies have shown that the presence of natural antioxidants from various aromatic and medicinal plants is closely related to the reduction of chronic diseases such as DNA damage, mutagenesis, and carcinogenesis (*30*−*38*). Essentially, antioxidants inhibit free radical propagation in biological systems. To characterize such a property in medicinal plants or other materials, measurement of the antioxidant capacity is widely used. Therefore, the aim of this study is to investigate the antioxidant capacity of VO and identify its antioxidant components. In this study, the antioxidant capacity of VO was

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investigated using two complimentary in vitro assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical scavenging assay and the Fe^{2+}-metal chelating assay. The radical scavenging activity of the crude VO was compared with that of commercial standard antioxidants, butylated hydroxytoluene (BHT) and α-tocopherol, through the DPPH• free radical scavenging assay. Meanwhile, the metal chelating capacity between VO and ethylenediaminetetraacetic acid (EDTA) was compared. Moreover, some VO components showing strong antioxidant activity were further separated from the crude VO by silica gel column chromatography and Spherisorb silica HPLC and identified by GC-MS.

MATERIALS AND METHODS

Materials and Chemicals. The crude VO was purchased from Good Scents Co. (Oak Creek, WI). Dry silica gel (70−230 mesh, 60 Å), DPPH•, α-tocopherol, and BHT were obtained from Sigma Chemical Co. (St. Louis, MO). Ferrous chloride and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine) were purchased from Fluka Chemical Co. (Milwakee, WI). EDTA and all HPLC analytical grade solvents were from Fisher Scientific (Suwanee, GA). α- and β-vetivones were kindly provided by Professor Ekkhard Winterfeldt (Institut für Organische Chemie, Technische Univesität, Berlin).

Fractionation and Identification of Antioxidants from VO. *Silica Gel Column Chromatography.* A glass column (60 × 2.5 cm) packed with silica gel (70−230 mesh, 60 Å) and maintained at 25 °C with a jacket was equilibrated with hexane. Three milliliters of the crude VO dissolved in hexane was loaded and eluted by multistep solvent gradients as follows: hexane, hexane/dichloromethane (DCM) (7:3), hexane/DCM (5:5), hexane/DCM (3:7), DCM, DCM/methanol (7:3), DCM/methanol (5:5), DCM/methanol (3:7), and methanol. The flow rate was 10 mL/min, and each collected fraction was 12 mL. After the open-column separation by silica gel column chromatography, those VO fractions bearing strong free radical scavenging activity determined by DPPH• assay were pooled for further analyses.

HPLC Separation. A Spherisorb silica column (250 × 4.6 mm, 5 μm; Waters, Milford, MA) was connected with a Shimadzu LC-10AT HPLC system (Kyoto, Japan) and equilibrated with hexane and DCM for separation of strong nonpolar compounds and weak nonpolar compounds, respectively. Fifty microliters of the pooled samples was injected into the HPLC column and eluted at a flow rate of 1 mL/min by linear gradient of DCM or methanol from 0 to 20% at 5−15 min and then by the linear gradient to 100% to desorb weakly polar compounds and polar compounds, respectively. All 1-mL fractions of the eluant were collected after passing through a Shimadzu SPD-M10V photodiode array detector (Kyoto, Japan). The scan range of absorbance for the eluant was from 200 to 300 nm.

GC-MS Identification. The Shimadzu's GC-MS system consisting of a GC-17A with a QP5050 mass spectrometer (Kyoto, Japan) was installed with a DB-5 capillary column (60 m × 0.25 mm, thickness = 0.25 μm; J&W Scientific, Folsom, CA) for all chemical quantitative and qualitative analysis in this experiment. The oven temperature was programmed from 60 to 240 °C at the ramp of 3 °C/min and held at 240 °C for 10 min. The injector and ion source temperatures were set at 200 and 250 °C, respectively. The detector voltage was 70 eV, and the MS spectra were scanned in the mass range of *m*/*z* 43−350. Helium was used as the carrier gas, and the column flow rate was 1.1 mL/min. Two microliters of each fraction containing the partially purified and separated antioxidants from the crude VO was injected into the capillary column with a split ratio of 2:1. Identification of compounds was based on comparison of their mass spectra and retention indices (RIs) with that of the Wiley and NIST mass spectral databases, the previously published RIs, and the authentic standards. RIs were calculated using a series of *n*-alkanes (C_8−C_{30}).

Antioxidative Capacity. The antioxidant capacities of VO and its separated antioxidative components were determined according to two methods: the DPPH• free radical scavenging assay and the metal chelating assay. Two standard antioxidants (i.e., BHT and α-tocopherol) were used as the control for the DPPH• free radical scavenging assay, whereas EDTA was used as the control for the metal chelating assay.

Table 1. Antioxidant Capacities of the Crude VO and Three Standard Antioxidants

	antioxidant capacity (EC_{50}, mg/mL)	
	scavenging activity	metal chelating activity
crude vetiver oil	7.79 ± 0.25	511.26 ± 70.53
BHT[a]	0.02 ± 0.00	
α-tocopherol[a]	0.02 ± 0.00	
EDTA[b]		0.024 ± 0.00

[a] BHT and α-tocopherol were used as standards for DPPH• free radical scavenging assay. [b] EDTA was a standard for metal chelating assay.

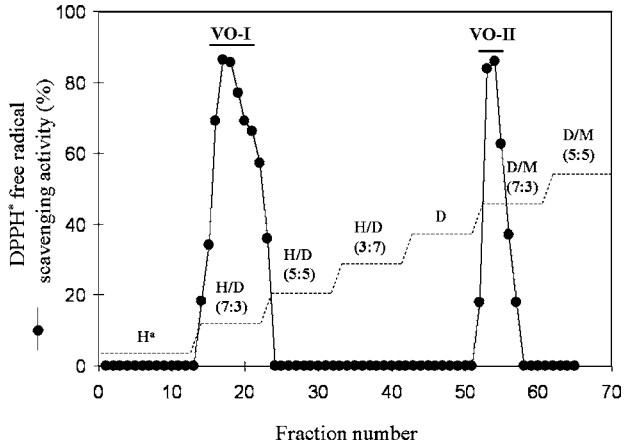

Figure 1. Free radical scavenging activity of the fractions of VO separated by silica gel open column chromatography. Antioxidant activities of all collected fractions were determined by the DPPH• assay. H, D, and M represent hexane, DCM, and methanol, respectively.

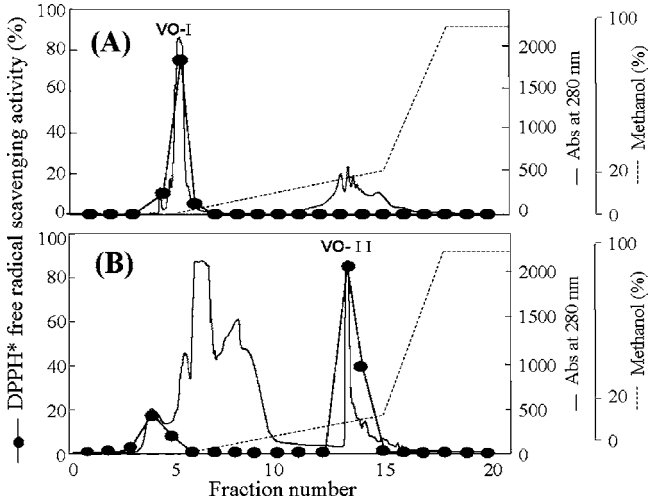

Figure 2. Chromatograms of VO-I (**A**) and VO-II (**B**) separated by Spherisorb silica HPLC and their corresponding free radical scavenging activities.

(a) DPPH• Free Radical Scavenging Assay. To determine the free radical scavenging activity of the crude VO and its components separated by silica gel column chromatography and Spherisorb silica HPLC, the DPPH• free radical scavenging assay reported by Yamaguchi et al. (*39*) with minor modification was used. The reaction mixture containing 0.1 mL of sample dissolved in methanol, 0.3 mL of methanol, and 0.4 mL of 0.3 mM DPPH• reagent dissolved in methanol was vigorously shaken and incubated in the darkness at room temperature for 10 min. After incubation, the absorbance of the reaction mixture was measured spectrophotometrically at 517 nm. The scaveng-

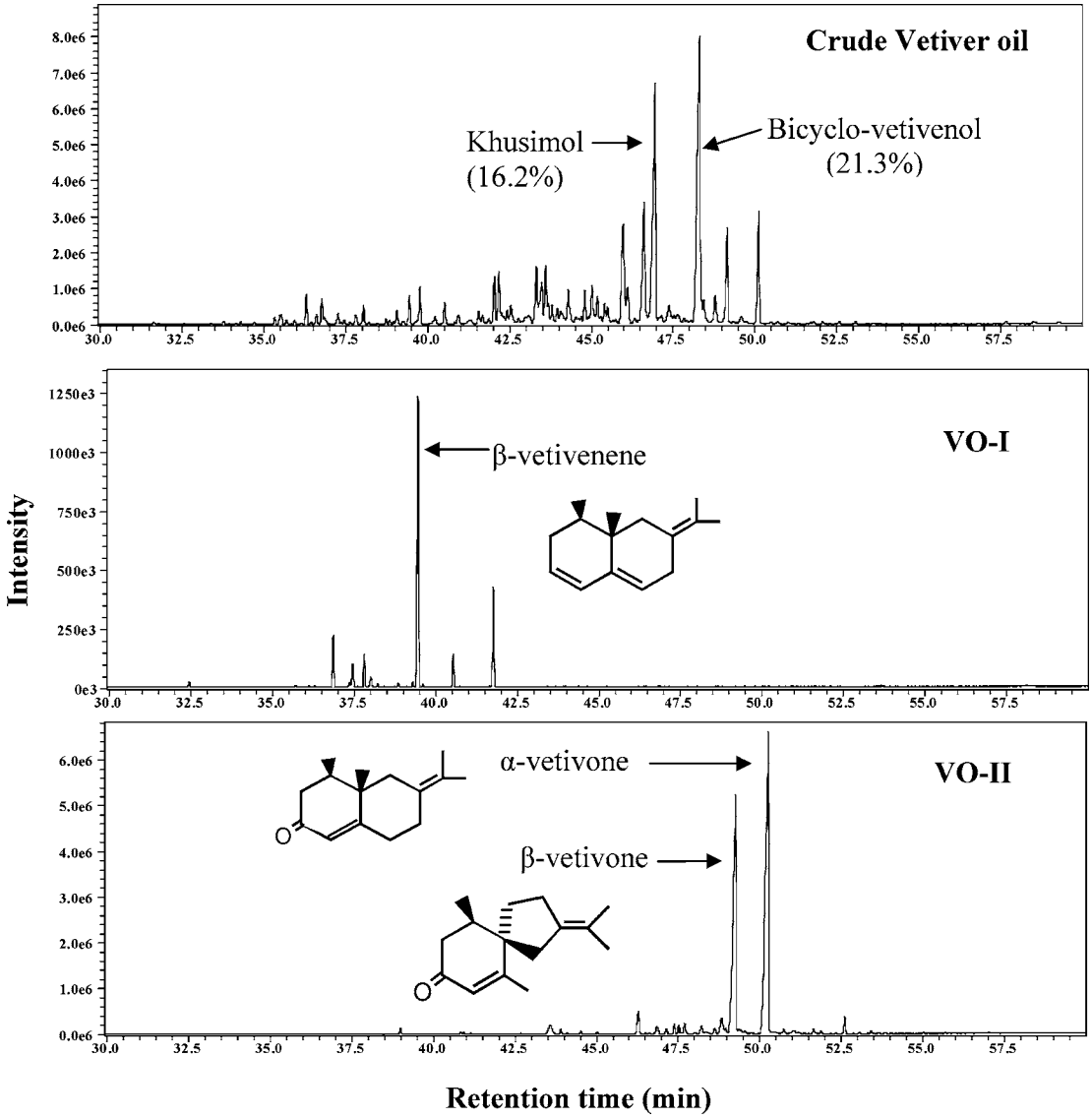

Figure 3. Gas chromatographic profiles of the crude VO, VO-I, and VO-II and identification of their major constituents. A DB-5 capillary column was installed in a Shimadzu GC-17A instrument that was also connected to a QP 5050 MS detector. The GC oven temperature was programmed from 60 to 240 °C at the rate of 3 °C/min and held at 280 °C for 10 min. The injector and ion source temperatures were 200 and 250 °C, respectively.

ing effect of DPPH· free radical was calculated by using the following equation:

$$\text{scavenging effect (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100 \quad (1)$$

(b) Metal Chelating Assay. The metal chelating effect of the crude VO and its components was determined according to the ferrous ion chelating assay modified from the method of Dinis et al. (*40*). A reaction solution composed of 600 μL of the sample and 40 μL of 2 mM $FeCl_2$ was activated by the addition of 80 μL of 5 mM ferrozine. After vortex, the reaction mixture was incubated at room temperature for 10 min, and its chelating activity was spectrophotometrically measured at 562 nm. The metal chelating effect was calculated by using the following equation:

$$\text{chelating effect (\%)} = \left(1 - \frac{\text{absorbance of sample at 562 nm}}{\text{absorbance of control at 562 nm}}\right) \times 100 \quad (2)$$

The EC_{50} value used to evaluate antioxidant capacities of the crude VO and standards was the effective concentration at which DPPH· radicals were scavenged and metal ions were chelated by 50%, respectively.

Statistical Analysis. The data of the antioxidant activities of the crude VO and its components were subjected to analysis of variance (ANOVA) and least significant difference (LSD at $p < 0.05$) tests by the SAS software for Windows, version 8 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Determination of the Antioxidant Activity of Crude VO. The antioxidant capacity of the crude VO was initially determined by the DPPH· free radical scavenging assay and the metal chelating assay and compared with those of two common antioxidants (e.g., BHT and α-tocopherol). Results are shown in **Table 1**. In the DPPH· free radical scavenging assay, the EC_{50} value of the crude VO dissolved in methanol was ~7.8 mg/mL, at which the scavenging activity was comparable to that of 0.02 mg/mL of BHT and α-tocopherol. However, in the ferrous ion metal chelating test, the inhibitive capacity of the VO against the metal chelating was much lower than that of a standard metal chelating agent, EDTA. The EC_{50} value of the crude VO was 511 mg/mL, whereas that of EDTA was 0.02 mg/mL at the same reaction condition. The results shown in **Table 1** indicate that the crude VO is moderately competitive

as a free radical scavenging agent to the well-known synthetic antioxidants such as BHT and α-tocopherol, but it may not be a good material to prevent free radical reactions initiated by metal ions.

Separation and Identification of Antioxidants from Crude VO. Because the crude VO has shown a strong antioxidant capacity in the DPPH• test, the inherent antioxidants in the crude VO were then separated sequentially with silica gel open-column chromatography and silica gel HPLC and finally identified by GC-MS.

When the crude VO was separated by the silica gel open column chromatography using a stepwise solvent elution method with different solvents such as hexane, dichloromethane, and methanol, a total of 65 fractions were collected. As shown in **Figure 1**, there were two strong antioxidant peaks from the DPPH• assay, namely, VO-I and VO-II. VO-I included the fractions 13−24 separated by a solvent mixture of hexane/DCM (7:3, v/v), which suggested that VO-I contained the strong/medium nonpolar compounds. VO-II contained fractions 51−58 that were separated by a mixture of DCM/methanol (7:3, v/v), which hinted that VO-II might contain some weakly polar and/or polar compounds. The strengths of the antioxidant activities of both VO-I and VO-II were similar, with the antioxidant activity values close to ∼86% in the DPPH• test. For further separation, all fractions within VO-I and VO-II were pooled and separated by the Spherisorb silica HPLC column. Chromatograms and the antioxidant activities measured by the DPPH• assay of each of both pooled fractions are shown in **Figure 2**. Major components of VO-I in hexane were eluted at low DCM concentration in fraction 5 that also exhibited a high free radical scavenging activity at 75% (**Figure 2A**). As shown in **Figure 2B**, most compounds of VO-II dissolved in DCM were eluted by 10% methanol and had a weak antioxidant activity. In contrast, fraction 13 in the same HPLC profile (**Figure 2B**) separated at 16% methanol possessed a very strong free radical scavenging activity of 85%. Thus, both fraction 5 of VO-I and fraction 13 of VO-II were selected for further chemical separation and identification by GC-MS.

Identification of Antioxidants in VO-I and VO-II Separated by Spherisorb Silica HPLC. The antioxidant constituents in fraction 5 of VO-I and fraction 13 of VO-II were further characterized by GC-MS. As shown in **Figure 3**, the crude VO had over 160 compounds including major components such as khusimol (∼16.2%) and bicylovetivenol (∼21.3%) accounting for 37.5% of the total amount of the crude VO. Khusimol and bicyclovetivenol, however, were not the targeted components in both pooled fractions because low free radical scavenging activity was shown, which suggested that these two major compounds did not play an important role in scavenging free radicals.

Further separation and identification of compounds in the two fractions revealed that there were seven and two compounds in fraction 5 of VO-I and fraction 13 of VO-II, respectively. β-Vetivenene, another major compound in fraction 5 and both β-vetivone and α-vetivone in fraction 13 were identified by comparing their RIs and mass spectra with those in a volatile oil library (*41*) and the Wiley and NIST mass spectral databases, as well as with the standard compounds under the same experimental conditions.

Although six other compounds besides β-vetivenene were in fraction 5, they were also detected in fractions 4 and 6 separated by the Spherisorb silica HPLC column. However, their free radical scavenging activities were much weaker. Thus, β-vetivenene might be the only free radical scavenging agent in fraction 5. Due to the fact that the content of β-vetivenene in the crude VO accounted for only ∼1.2%, β-vetivenene was cautiously considered as an important antioxidant in VO. Unlike β-vetivenene, both β-vetivone and α-vetivone identified in fraction 13 of the VO-II were weakly polar compounds, and their contents in the crude VO were about 5.2 and 6.6%, respectively, which were equivalent to about 4- and 5-fold higher contents than that of β-vetivenene.

Although these three compounds have shown strong antioxidant activities in our preliminary screening, their concentration−activity relationship unfortunately could not be established due to the lack of enough and available standards. Spreitzer et al. (*42*, *43*) reported the novel means to chemically synthesize vetivones, but so far there are still no commercial vetivones available. Nevertheless, our present results certainly suggest that the crude VO and some of its inherent antioxidant constituents could be considered as novel natural antioxidants, which might have alternative potential applications. Moreover, if a large quantity of β-vetivone and α-vetivone can be easily separated from the crude VO by chromatographic techniques or cost-effectively synthesized, it is believed that vetivones will acquire more practical applications in other areas besides their current utilization as effective termiticides (*19*) and cosmeceuticals.

In summary, the most intriguing result of our study was the demonstration of VO and some of its inherent bioactive terpenoids having strong reducing power through the DPPH• free radical scavenging assay. To our knowledge, such results have never been reported. Moreover, chemical synthesis of vetivones and investigation of their anticancer functionalities are underway in our laboratory.

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