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Identification of an Antioxidant, Ethyl Protocatechuate, in Peanut Seed Testa

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The antioxidant activity and identification of the antioxidant component of peanut seed testa were investigated. The antioxidant activity of peanut seed testa was studied in the linoleic acid model system by using the ferric thiocyanate method. Among the five organic solvent extracts, the ethanolic extracts of peanut seed testa (EEPST) produced higher yields and stronger antioxidant activity than other organic solvent extracts. EEPST was separated into 17 fractions on silica gel column chromatography. Fraction 17, which showed the largest yield and significant antioxidant activity, was separated by thin-layer chromatography. Four major antioxidative subfractions were present. Subfraction 17-2 was found to be effective in preventing oxidation of linoleic acid. This subfraction was further fractionated and isolated and characterized by UV, MS, IR, and ^{1}H NMR techniques. The active compound was identified as ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester).

KEYWORDS: Peanut seed testa; antioxidant; identification; structure elucidation; liquid chromatography; ethyl protocatechuate

INTRODUCTION

Antioxidants are used to preserve food by retarding rancidity, discoloration, or deterioration due to autoxidation. Synthetic antioxidants are widely used because they are effective and cheaper than natural types. However, there has been growing concern over their safety and toxicity (*1*). In addition, many epidemiological studies have strongly suggested that there is a correlation between intake of antioxidant-rich foods and low mortality (*2*). Hence, it is important to determine the natural antioxidant source. However, natural antioxidants such as tocopherol and ascorbic acid that are used as alternatives to replace synthetic antioxidants are much less effective than synthetic antioxidants (*3*). Therefore, the identification, development, and utilization of antioxidants from natural sources are beneficial and desired.

Soybean products, oat products, crude vegetable oils, amino acids, nordihydroguaiaretic acid, fruits, nuts, seeds, leaves, roots, barks, and Maillard reaction products, etc., have thus been exploited as potential sources of natural antioxidants (*4*). Peanut is a principal agricultural plant in the world. The antioxidative property of peanut kernels has been investigated. The flavonoid dihydroquercetin, extracted from peanut kernels, exhibited antioxidant activity (*5*). In our previous studies (*6*−*13*), peanut hulls were found to exhibit marked antioxidant activity and antimutagenic effect, and an antioxidant component of methanolic extracts of peanut hulls was identified as luteolin (*6*).

The byproducts of agroindustries such as seed testa, hulls, and peels are wasted or used as feedstuffs and fertilizers. Recently, the investigation of antioxidant components extracted from hulls (*6*−*17*), coats (*18*, *19*), and peels (*20*) has been reported.

Peanuts consists of peanut kernels, peanut seed testa, and peanut hulls. The literature has demonstrated that peanut kernels and peanut hulls contain antioxidant components, and the antioxidant properties of peanut have been studied (*5*−*13*). In addition, a flavone and a flavanone in peanut seed testa were purified by Masquel and Blanquet in 1948 (*21*, *22*); however, no reports on the antioxidant activity of peanut seed testa have been made so far. Whether the peanut seed testa exhibits antioxidant activity remains unclear. Therefore, investigation of the antioxidant activity of peanut seed testa is needed.

In the present study, we determined the antioxidant activity in the linoleic acid of peanut seed testa by using the ferric thiocyanate method model system. Major antioxidant principles of peanut seed testa were also isolated and identified.

MATERIALS AND METHODS

Materials. Peanut seed testae, Spanish-type, obtained from Sand Kong Corp. (Yunlin, Taiwan), were stored at 4 °C until used. Linoleic acid, ammonium thiocyanate, ferrous chloride, and butylated hydroxy-

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anisole (BHA) where purchased form E. Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Extraction. The peanut seed testae (5.0 g) were extracted overnight with 200 mL of methanol, ethanol, acetone, hexane, and ethyl acetate, respectively, in a shaking incubator at room temperature. The extracts were filtered, and the extraction was repeated. The combined filtrates were evaporated to dryness in vacuo and weighed to determine the yield of soluble constituents.

Antioxidant Activity in a Linoleic Acid System. Antioxidant activity assay was carried out by using the linoleic acid system. Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (0.2804 g) and Tween 20 (0.2804 g) in potassium phosphate buffer (50 mL, 0.05 M, pH 7.4). A reaction solution containing extracts (0.2 mL, 5.0 mg/mL), linoleic acid emulsion (2.5 mL), and potassium phosphate buffer (2.3 mL, 0.2 M, pH 7.0) was mixed with a homogenizer. The reaction mixture was incubated at 37 °C in the dark, and the degree of oxidation was measured according to the thiocyanate method (*23*), by sequentially adding ethanol (4.7 mL, 75%), ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL), and ferrous chloride (0.1 mL, 0.02M). After the mixture had been stirred for 3 min, the peroxide value was determined by reading the absorbance at 500 nm, and the inhibition percent of linoleic acid peroxidation was calculated as (%) inhibition $=100 -$ [(absorbance increase of sample/absorbance increase of control) $\times$ 100]. All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

Silica Gel Column Chromatography. The ethanolic extracts of peanut seed testa (EEPST) were fractionated by silica gel column (9.5 cm diameter and 125 cm height, 70−230 mesh particle size, E. Merck) chromatography. A 458.3 g sample of EEPST was dissolved in ethanol and evaporated to powder. The powder sample was mixed with silica gel and introduced to the column. CH_2Cl_2, CH_2Cl_2/ethanol (10:1, v/v), and CH_2Cl_2/ethanol (5:1, v/v) were used as eluant solvents, respectively. Silica gel chromatography of the material gave 17 fractions. Each fraction was evaporated to dryness and weighed to determine the yield and then redissolved in ethanol for measuring the antioxidant activity (*23*).

Identification of Phenols. Identification of the phenols of each fraction was according to the method of Pratt and Miller (*5*). The same volumes of ferric chloride (1%) and potassium ferricyanide (1%) were mixed and sprayed, making an orange-brown solution with no blue color, and sprayed. Phenols give an immediate blue color.

Purification of Active Antioxidative Component. Of the 17 fractions, fraction 17 showed the highest yield and marked antioxidant activity, determined by using the thiocyanate method (*23*), and was used for further purification on a silica gel column chromatography. Fraction 17 (16.54 g) was loaded on the top of the column. The solution of chloroform/acetone (5:1, v/v) was used as the elution solvent.

Silica gel chromatography of fraction 17 yielded four subfractions, named subfractions 17-1, 17-2, 17-3, and 17-4, respectively. The antioxidant activity of each subfraction was determined by using the thiocyanate method (*23*). Subfraction 17-2 showed marked antioxidant activity and was further investigated. Acetone solvent was added to subfraction 17-2, and some material was crystallized. After the crystal called subfraction 17-2-1 was removed, the soluble components in acetone were named subfraction 17-2-2.

Test of Antioxidant Activity. The antioxidant activity of subfractions 17-2-1 and 17-2-2 was examined by a thin-layer chromatography (TLC) methodology on silica gel plates (20 $\times$ 20 cm, F_{254}, 0.25 mm, E. Merck) using chloroform/acetone (9:1, v/v) as a mobile phase. Antioxidants on TLC plates were detected using the carotene spray according to the method of Pratt and Miller (*5*). Nine milligrams of β-carotene was dissolved in 30 mL of chloroform. Two drops of linoleic acid and 60 mL of ethanol were added to the β-carotene/chloroform solution. This solution was sprayed on chromatograms spotted with subfractions 17-2-1 and 17-2-2. After spraying, the background color of of the chromatograms was bleached because the visual color of β-carotene had disappeared due to oxidation of β-carotene. Spots in which a yellow color persisted possessed antioxidant activity.

Instrumental Analysis of Active Antioxidative Component. *UV Spectrophotometry.* The UV absorption spectrum of the active antioxidative component in methanol was recorded. A Hitachi UV-3210 spectrophotometer (Hitachi, Tokyo, Japan) was used.

IR Spectrometry. IR spectral data were recored in potassium bromide (KBr) disks with a Jasco IR Report-100 unfrared spectrophotometer (Jasco, Tokyo, Japan). One milligram of KBr granules was ground into fine powder and made into a thin transparent tablet. The dry sample (0.3 mg) was dissolved with 5 drops of chloroform. The sample solution was spotted on a KBr tablet and then blown to dryness.

Nuclear Magnetic Resorance (NMR) Spectrometry. NMR spectra for ^{1}H at 200 MHz were recorded on a Bruker AC-200 spectrometer (Bruker, Karlsruhe, Germany) at room temperature (28 °C). Chemical shifts were recorded relative to tetramethylsilane (TMS) as an internal standard.

Mass Spectrometry. Mass spectra were obtained on a Finnigan LCQ duo spectrometer (Finnigan, San Jose, CA) by liquid chromatography−mass spectrometry (e: 40%).

Table 1. Yield and Antioxidant Activity of Peanut Seed Testa Extracted with Various Solvents

sample[a]	yield[b] (mg)	antioxidant activity[c−e] (%)
MEPST	212.4	93.5 ± 2.0c
EEPST	252.8	95.8 ± 0.1b
AEPST	199.0	3.0 ± 2.4e
HEPST	35.1	3.0 ± 0.4e
EAEPST	90.4	0.3 ± 0.4f
BHA		99.1 ± 3.6a
Toc		76.1 ± 0.4d

[a] MEPST, methanol extracts of peanut seed testa; EEPST, ethanol extracts of peanut seed testa; AEPST, acetone extracts of peanut seed testa; HEPST, hexane extracts of peanut seed testa; EAEPST, ethyl acetate extracts of peanut seed testa; BHA, butylhydroxy anisole; Toc, tocopherol. [b] Based on 5.0 g of peanut seed testa for each solvent (200 mL). [c] Antioxidant activity of each sample (200 ppm) was determined by thiocyanate method. [d] Values are mean ± standard deviation of three replicate analyses. [e] Means within a column with the same letters are not significantly different ($p > 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the yields and antioxidant activity of peanut seed testa extracted with various solvents. Among the solvent extracts, ethanol extracts produced the highest yields, followed by methanol extracts, acetone extracts, ethyl acetate extracts, and hexane extracts. The results indicate that the polar solvents on the extraction were more efficient than less polar solvents. As shown in **Table 1**, the antioxidant activities of various solvent extracts were compared with commercial antioxidants such as *dl*-α-tocopherol (Toc) and BHA. Among the five solvent extracts, EEPST at 200 ppm showed the strongest antioxidant activity. In addition, the antioxidant activity of EEPST at 200 ppm was stronger than 200 ppm of Toc. From **Table 1**, it is evident that the antioxidant activity of polar solvent extracts was markedly stronger than that of less polar solvent extracts, which showed hardly any antioxidant activity.

Of the five solvent extracts, EEPST exhibited the highest yield and the strongest antioxidant activity. Ethanol was, therefore, chosen for extraction for antioxidant isolation and identification. EEPST was separated into 17 major fractions on silica gel column chromatography. Fractions 1−9 and fractions 10−17 were chromatographed on a silica gel plate and eluted with CH_2Cl_2 and CH_2Cl_2/CH_3OH (25:1, v/v), respectively, to obtain different R_f values (data not shown), indicating that each fraction contained different components. The yield, antioxidant activity, and phenolic characteristic of each fraction are shown in **Table 2**. The antioxidant activity of fraction 14 was the strongest of all the fractions; however, its yield was less than that of fraction 17. The yield of fraction 17 was higher than that of other

Table 2. Some Characteristics of Different Fractions of Ethanolic Extracts from Peanut Seed Testa Separated on Silica Gel Column Chromatography

fraction[a]	yield (g)	antioxidant activity[b,c] (%)	blue color[d]
1	0.063	33.9 ± 0.00f	−
2	1.087	2.1 ± 0.00jk	−
3	0.801	0.3 ± 0.30jk	−
4	4.755	0.5 ± 0.40jk	+
5	0.219	5.7 ± 1.60h	+
6	0.314	2.7 ± 0.50jk	+
7	0.278	2.7 ± 1.00jk	−
8	1.217	0.0 ± 0.00jk	−
9	0.383	0.8 ± 0.80jk	+
10	0.039	3.9 ± 1.80j	−
11	8.272	8.4 ± 0.00h	−
12	1.173	79.8 ± 0.00d	−
13	2.800	80.8 ± 040d	+
14	0.460	88.6 ± 0.00c	−
15	1.246	78.0 ± 1.90d	−
16	14.448	19.6 ± 3.60g	−
17	16.539	78.6 ± 0.00d	+
EEPST		95.8 ± 1.00b	
Toc		71.1 ± 0.20e	
BHA		99.1 ± 0.00a	

[a] EEPST, ethanolic extract of peanut seed testa; BHA, butylhydroxy anisole; Toc, tocopherol. [b] Antioxidant activity of each sample (200 ppm) was determined by thiocyanate method. [c] Means within a column with the same letters are not significantly different ($p > 0.05$). [d] Each fraction was reacted with ferric chloride–potassium ferricyanide, and phenolic compounds gave a blue color.

fractions. No significant difference ($p > 0.05$) in antioxidant activity was found among fractions 12, 13, 15, and 17. Obviously, fraction 17 with the highest yield and remarkable antioxidant activity was further separated to obtain the most active antioxidant component; the other fractions remain to be further investigated.

Previous studies suggested that polyphenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid oxidation. To elucidate the causes of antioxidant characteristics of the fractions, it is essential to evaluate whether the antioxidant activity of the fractions is related to phenolic compounds. Ascending TLC on silica gel plates developed in chloroform/methanol (9:1, v/v), fractions 4−6, 9, 13, and 17 have positive reactions to ferric chloride−potassium ferricyanide sprays. These results indicate these fractions to be phenolics. However, other fractions with antioxidant activity gave negative reactions to ferric chloride−potassium ferricyanide sprays. This indicates that the phenolic compounds are not the only factor in the consideration of the antioxidant activity of EEPST. Although EEPST exhibited marked antioxidant activity, all isolated fractions showed lower antioxidant activity than EEPST. Possible synergism of phenolics with one another or with other components present in EEPST may be responsible for this observation.

Figure 1 shows the antioxidant activity of fraction 17 separated on silica gel column chromatography. Fraction 17 was separated into four major subfractions. The antioxidant activity was in the following descending order: subfraction 17-1 (58.5%) > subfraction 17-2 (37.5%) > subfraction 17-4 (34.5%) > subfraction 17-3 (22.2%). All four subfractions showed antioxidant activity, although the activity of all four subfractions was less than that of fraction 17 (78.6%). In other words, the antioxidant activity of fraction 17 was greater than the sum of its subfractions. This may be due to the fact that the synergism of each subfraction with one another may contribute to this observation. Because the antioxidant activities of subfractions 17-1 and 17-2 were stronger than those of subfractions 17-3 and 17-4, subfractions 17-1 and 17-2 were further investigated. Subfractions 17-1 and 17-2, loaded on a thin-layer chromatograph, were developed on silica gel using chloroform/acetone (9:1, v/v) as a mobile phase. Subfraction 17-1 was fractionated into 15 spots. The R_f values of all spots fractionated from subfraction 17-1 were very close, and the yield of each spot was too small to be purified. Hence, the isolation and identification of subfraction 17-1 remain to be further investigated. On the other hand, subfraction 17-2 was fractionated into two spots, named subfractions 17-2-1 and 17-2-2, suggesting that subfraction 17−2 is available for easier isolation and structural elucidation than subfraction 17-1. Hence, subfractions 17-2-1 and 17-2-2 fractionated from subfraction 17-2 were further investigated.

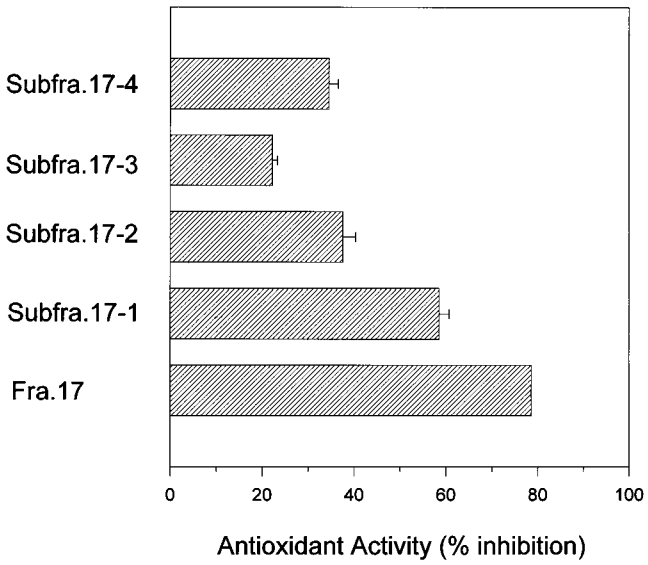

Figure 1. Antioxidant activity of fraction 17 separated by silica gel column chromatography. Antioxidant activity of each sample (200 ppm) was determined by thiocyanate method. Fra., fraction; Subfra., subfraction.

The antioxidant activities of subfractions 17-2-1 and 17-2-2 were determined by using a β-carotene/linoleate spray system. Subfraction 17-2-2 was more effective in preventing the oxidation of β-carotene than subfraction 17-2-1. Therefore, the chemical structure of subfraction 17-2-2 was determined by instrumental analysis as follows. Subfraction 17-2-2, colorless granules, had a molecular weight of *m*/*z* 183 ($M^+ + 1$) by liquid chromatography mass spectrum. The UV spectrum, with absorption bands at 219.4 (sh), 260.0, and 292.6 nm, and the IR spectrum, with bands at 1715 and 1605 cm^{-1}, showed the structure of the compound to be an esteryl benzene ring nucleus (*24*).

In the ^1H NMR spectrum, ABX pattern signals at δ 6.91 (1H, d, $J = 8.6$ Hz, H-5), δ 7.57 (1H, dd, $J = 2.0$, 8.6 Hz, H-6), and δ 7.58 (1H, d, $J = 2.0$ Hz, H-2) indicated the presence of three substituents on the benzene ring. Two hydroxyl groups at δ 6.03 and 6.05 (each 1H, br s, D_2O exchangeable) and an ethyl group at δ 1.37 (3H, t, $J = 7.0$ Hz) and δ 4.33 (2H, dd, $J =$ 4.4, 7.0 Hz) also appeared in ^1H NMR spectrum. In the mass spectrum, the fragment ions at *m*/*z* 137 for [$M^+ -$ OEt] and 111 for [$M^+ -$ COOEt] were supported by the presence of a carboxy ethyl substituent; therefore, two hydroxyl groups were substituted on the benzene ring. On the basis of these data, the structure of subfraction 17-2-2 was identified as ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester, **Figure 2**) (*25*).

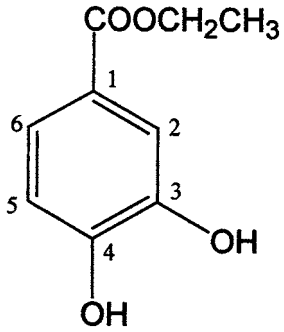

Figure 2. Structure of ethyl protocatechuate.

In conclusion, EEPST exhibited antioxidant activity in a linoleic acid model system. Separation of the crude extracts afforded 17 fractions; the antioxidant activity of each fraction was somewhat less than that of the original extracts. Fraction 17 showed the highest yield and marked antioxidant activity. TLC separation of this fraction allowed the isolation of the antioxidant component of peanut seed testa, which was identified as ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester).

LITERATURE CITED

(1) Ito, N.; Hirose, M.; Fukushima, S.; Tsuda, H.; Shirai, T.; Tatematsu, H. Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinoginesis. *Food Chem. Toxicol.* **1986**, *24*, 1099−1102.

(2) Rice-Evans, C. A.; Sampson, J.; Bramley, P. M.; Hollowa, D. E. Why do we expect carotenoids to be antioxidants in vivo. *Free Radical Res.* **1997**, *26*, 381−398.

(3) Wanasundara, U.; Amarouicz, R.; Shahidi, F. Isolation and identification of an antioxidatire componect in canola meal. *J. Agric. Food Chem.* **1994**, *42*, 1285−1290.

(4) Schuler, P. Natural antioxidants exploited commercial. In *Food Antioxidants*; Hudson, B. J. F., Ed.; Elsevier Applied Science: London, U.K., 1990; pp 127−135.

(5) Pratt, D. E.; Miller, E. E. A flavonoid antioxidant in Spanish peanuts. *J. Am. Oil Chem. Soc.* **1984**, *61*, 1064−1067.

(6) Duh, P. D.; Yeh, D. B.; Yen, G. C. Ertraction and identification of an antioxidative component from peanut hulls. *J. Am. Oil Chem. Soc.* **1992**, *69*, 814−818.

(7) Yen, G. C.; Duh, P. D.; Tsai, C. L. Relationship between antioxidant activity and maturity of peanut hulls. *J. Agric. Food Chem.* **1993**, *41*, 67−70.

(8) Yen, G. C.; Duh, P. D. Antioxidative properties of methanolic extracts from peanut hulls. *J. Am. Oil Chem. Soc.* **1993**, *70*, 383−386.

(9) Yen, G. C.; Duh, P. D. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *J. Agric. Food Chem.* **1994**, *42*, 629−632.

(10) Yen, G. C.; Duh, P. D. Antioxidant activity of methanolic extracts of peanut hulls from various cultivars. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1065−1067.

(11) Duh, P. D.; Yen, G. C. Changes in antioxidant activity and components of methanolic extracts of peanut hulls irradiated with ultraviolet light. *Food Chem.* **1995**, *54*, 127−131.

(12) Yen, G. C.; Duh, P. D. Antimutagenic effect of methanolic extracts from peanut hulls. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1698−1700.

(13) Duh, P. D.; Yen, G. C. Antioxidant efficacy of methanolic extracts of peanut hulls in soybean and peanut oils. *J. Am. Oil Chem. Soc.* **1997**, *74*, 745−748.

(14) Duh, P. D.; Yen, W. J.; Du; P. C. Antioxidant activity of mung bean hulls. *J. Am. Oil Chem. Soc.* **1997**, *74*, 1059−1063.

(15) Duh, P. D.; Du, P. C.; Yen, G. C. Action of methanolic extracts of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. *Food Chem. Toxicol.* **1999**, *37*, 1055−106.

(16) Watanabe, M.; Ohshita, Y.; Tsushida, T. Antioxidant compounds from buckwheat (*Fagopyrum esculentum* Moench) hulls. *J. Agric. Food Chem.* **1997**, *45*, 1039−1044.

(17) Zing, Y.; White, P. Identification and function of antioxidants from oat groats and hulls. *J. Am. Oil Chem. Soc.* **1997**, *74*, 303−307.

(18) Chang, L. W.; Yen, W. J.; Huang, S. C.; Duh, P. D. Antioxidant activity of sesame coat. *Food Chem.* **2002**, *78*, 347−354.

(19) Muamza, D.; Robert, R.; Sparks, W. Antioxidant derived from lentil and its preparation and uses. U.S. Patent US5762936, 1998.

(20) Larrauri, J. A.; Ruperez, P.; Saura-Calixto, F. Effect of drying temperature on the stability of polyphenols and antioxidant activity of red grape pomace peels. *J. Agric. Food Chem.* **1998**, *46*, 4842−4845.

(21) Masquelier, J.; Blanquet, P. Studies on the pigments of the peanut. A flavone. *Compt. Rend.* **1948**, *227*, 641−642.

(22) Masquelier, J.; Blanquet, P. Chemical studies of the pigments of the peanut. II The flavanone. *Bull. Soc. Fr.* **1948**, 1172−1175.

(23) Misuda, H.; Yasumoto, K.; Iwami, K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo to Shokuryo* **1966**, *19*, 210−214.

(24) Scott, A. I. *Interpretation of the Ultraviolet Spectra of Natural Products*; Scott, A. I., Ed.; Pergamon Press: London, U.K., 1964; pp 89−119.

(25) Chang, W. L.; Lee, S. S. Norneolignan and phenols from *Curculigo capitulata*. *Phytochemistry* **1998**, *49*, 2133−2136.

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