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Antioxidant activity of compounds isolated from the pyroligneous acid, *Rhizophora apiculata*

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Abstract

The polyphenols (CPAE II) was isolated from the dichloromethane extract of the pyroligneous acid, *Rhizophora apiculata* by simultaneous acid base and solvent extraction method. Its qualitative and quantitative composition was studied by gas chromatography mass spectroscopy (GC/MS) and out of 57 peaks, 52 compounds were identified, representing 95.47% of the total polyphenols. The CPAE II was then fractionated to four fractions (F1–F4) by means of thin layer chromatography and silica gel column chromatography with dichloromethane, dichloromethane/chloroform/ethyl acetate mixture (8:1:1; 4:3:3, v/v/v), and ethyl acetate, respectively. The antioxidant properties of the CPAE II and the fractions were evaluated. Among the four fractions, fraction 1 (F1) was the most potent in DPPH radical scavenging activity and molybdenum (VI) reducing power. It was subjected to further purification by means of silica gel column chromatography with hexane, hexane/diethyl ether mixture (9:1, 6:1, 3:1, v/v), and diethyl ether, respectively. 2,6-Dimethoxyphenol (syringol) and dihydroxybenzenes (catechol and 3-methoxycatechol) were isolated and identified by GC/MS, ^{1}H NMR, ^{13}C NMR spectral analyses, and confirmed by GC co-injection with authentic standards. Syringol, catechol and 3-methoxycatechol constitute 39.08, 4.21 and 1.10% of F1, respectively. Their antioxidant activities were evaluated by DPPH radical scavenging activity, ABTS radical cation scavenging activity, phosphomolybdenum and ferric reducing antioxidant power (FRAP). The trend in antioxidant capacity was similar in all the four assays, with dihydroxybenzenes > 2,6-dimethoxyphenols, although discrepancies in the ranking within the dihydroxybenzenes were present. These three compounds which showed significant antioxidant activities were isolated for the first time from the pyroligneous acid, *R. apiculata*.

Keywords: *Rhizophora apiculata*; Pyroligneous acid; Polyphenols extract; Antioxidant activities; Syringol; Catechol; 3-Methoxycatechol

1. Introduction

Polyphenols are secondary plant metabolites that have been reported to have anti-carcinogenic, anti-mutagenic and antioxidant activities. They can act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Rice-Evas, Miller, & Paganga, 1996). In recent years, evaluation of antioxidative activity of naturally occurring substances has been the focus of interest (Jayaprakasha, Jaganmohan Rao, & Sakariah, 2004). The growing interest in the substitution of synthetic antioxidant by natural ones has fostered research on plant sources and the screening of raw materials for identifying new antioxidants. Sources of natural antioxidants are primarily plant phenolics, that may occur in all parts of the plants such as fruits, vegetables, nuts, seeds, leaves, roots, and barks (Pratt & Hudson, 1990). Phenols found in coal and biomass pyrolysis liquids are important compounds of increasing interest as well (Churin et al., 1988). Special attention is focused on the extraction from the inexpensive or residual sources from agricultural industries (Moure et al., 2000).

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In Malaysia, pyroligneous acid or wood vinegar is the name of the crude condensate produced from the distillation of smoke generated in the process of making charcoal. The synonyms for pyroligneous acid include pyrolysis oils, pyrolysis liquids, wood liquids, liquid smoke, liquid wood, bio-oil, bio-crude oil and wood distillates. Chemically, pyroligneous acid is a complex mixture of water, guaiacols, catecols, syringols, vanillins, furan carboxaldehydes, isoeugenol, pyrones, acetic acid, formic acid, and other carboxylic acids. It also contains other major groups of compounds, including hydroxyaldehydes, hydroxyketones, sugars, carboxylic acids, and phenolics (Fengel & Wegener, 1983; Guille'n & Manzanos, 1999a; Guille'n & Manzanos, 2002; Piskorz, Scott, & Radlien, 1988; Shafizadeh, 1984).

Pyroligneous acid has been traditionally used as sterilizing agent, deodorizer, fertilizer, antimicrobial and growth promoting agent. Besides, it has also been used as a source of smoke flavours (Mohan, Pittman, & Steele, 2006). Smoke flavours are considered to be Generally Regarded As Safe (GRAS), so they can be used in foods as an additional barrier to prevent microbial growth at levels which comply with good manufacturing practice (Holley & Patel, 2005). Organisms which play an important role in the spoilage of foods, and bacteria of public health significant, are effectively inhibited by some wood smoke (Sunen, 1998). The composition of smoke has been extensively studied in recent years and more than 2000 compounds were identified (Guille'n & Ibargoitia, 1996a, 1996b, 1999; Guille'n & Manzanos, 1996, 1997, 1999a, 1999b, 2002; Guille'n, Manzanos, & Ibargoitia, 2001; Guille'n, Manzanos, & Zabala, 1995). These compounds belong to many different chemical classes: aldehydes; ketones; alcohols; acids; esters; furan and pyran derivatives; phenolic derivatives; hydrocarbons; nitrogen compounds. It was reported that the antimicrobial activity of pyroligneous acid is attributed to the presence of compounds like phenolic compounds, carbonyls and organic acids (Vitt, Himelbloom, & Crapo, 2001). Among them, the phenolic compounds probably represent the most important one both from the qualitative and quantitative point of view. The phenolic compounds, and in particular the methoxyphenols, have been considered the major contributors to smoke aroma and are responsible for the antimicrobial and antioxidant effects in smoked foods (Estrada-Munoz, Boyle, & Marsden, 1998; Faith, Yousef, & Luchansky, 1992; Guille'n & Ibargoitia, 1998; Sunen, Aristimuno, & Fernandez-Galian, 2003). Phenolic compounds also contribute to sensory properties of foods, particularly colour and astringency (Garrote, Cruz, Moure, Dominguez, & Parajo, 2004). In addition, smoking affects the organoleptic properties of food by imparting a characteristic flavour and taste, and by modifying the texture and the colour (Martinez, Salmeron, Guillen, & Casas, 2007; Toth & Potthast, 1984). It was reported that mayonnaises with added pyroligneous acid had a higher intensity of whiteness and colour tone than mayonnaises without pyroligneous acid (Wendin, Ellekjaer, & Solheim, 1999).

Besides its antimicrobial properties, previous work has been done and evidently shown that the dichloromethane extract of the pyroligneous acid of *Rhizophora apiculata* (CPAE) is a rich source of antioxidants, due to its high total phenolic content, superior free radical scavenging activity and ferric reducing power (Loo, Jain, & Darah, 2007). Our objectives in this study were to extract the polyphenols (CPAE II) from the dichloromethane extract of the pyroligneous acid, *R. apiculata* (CPAE), fractionate the polyphenols extract by silica gel column chromatography, thus obtained into several fractions, and to evaluate their antioxidant properties. In addition, the major antioxidant compounds in column chromatography fraction 1 (F1) were isolated and purified with various chromatographic techniques and, their structure identified by GC/MS, ^{1}H NMR, ^{13}C NMR spectral, and confirmed by GC co-injection with authentic standards. Their antioxidant activity were screened individually with some classical assays including DPPH radical scavenging activity, ABTS radical cation scavenging activity, phosphomolybdenum assay and ferric reducing antioxidant power (FRAP).

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), alpha-tocopherol, Trolox, ascorbic acid, 2-thiobarbituric acid (TBA), 2,2 azobis-(3-ethylbenzothiozoline-6-sulphonic acid) (ABTS), Folin Ciocalteu's reagent, Whatman No.1 filter paper, pottasium ferricyanide, ferric chloride, sodium carbonate, chloroform-*d*, ACS grade methanol and all analytical grade chemicals were purchased from Sigma (Sigma Aldrich GmbH, Sternheim, Germany). Silica gel-60 (230–300 mesh) was purchased from Fluka (Sigma-Aldrich, Switzerland), TLC plates (pre-coated of silica gel-60 F_{254}, 20 × 20 cm, coated on aluminium sheet) were purchased from Merck (Selangor Darul Ehsan, Malaysia).

2.2. Plant materials and extraction

2.2.1. Plant material

The raw pyroligneous acid of *R. apiculata* was obtained from Kuala Sepetang Charcoal village (4°50′N, 100°35′E) in the State of Perak, on the northwest coast of peninsular Malaysia. The sample was stored in the dark at below 20 °C before use.

2.2.2. Extraction of polyphenols by acid–base method

The extraction of polyphenols was carried out based on procedures described by Amen-Chen, Pakdel, and Roy (1997) with some modifications. A sample of dichloromethane extract of pyroligneous acid, CPAE (25 mL, 1.020 g/mL) was mixed with an sodium hydroxide solution (3 M) in a 1:1 ratio by weight in a 250 mL separatory funnel.

The funnel was shaken and two phases were separated on standing and the acidity of the resulting phases was measured. Neutral compounds will not react with acidic or basic extracting agents and will remain in the organic layer. While all the acidic compounds, both strong (carboxylic acid) and weak (phenols) acids will be extracted into the aqueous layer. The alkaline extraction of the solvent phase was repeated three times with a fresh sodium hydroxide solution. An aliquot of the aqueous phases was collected in a 500 mL separatory funnel and phenols were regenerated be acidifying the solution with aqueous sulphuric acid (3 M) to pH 2–3. Relatively strong organic acids were then extracted into aqueous layers using sodium bicarbonate. Approximately 260 mL of aqueous phase were obtained. A 50 mL of dichloromethane was then used as the organic solvent to recover the polyphenols extract, or CPAE II from the aqueous phase. Dichloromethane was selected, not only for its high efficiency in extracting polar compounds such as phenols, but also for its high volatility (Guille'n & Manzanos, 1996; Yrieix, Gonzalez, Deroux, Lacoste, & Leybros, 1996). However, dichloromethane is used for experimental purpose, but is not a recommended solvent for extraction to be used by the industry. The extraction was repeated five times with a 1:1 solvent/aqueous phase volume ratio.

2.2.3. Fractionation of the polyphenols extract

The solvent mixture (250 mL) of chloroform, ethyl acetate, and dichloromethane (4:5:6, v/v/v) was submitted to a silica gel column (1.5 cm internal diameter × 80 cm) which had been prepared by pouring a slurry of silica gel and solvent mixture into a chromatographic tube (1.5 cm internal diameter × 15 cm) and left overnight. Ten millilitres of the yellowish brown CPAE II (approximately 11.75 g) were then applied onto a silica gel (70 g, 230–400 mesh, Fluka) column and eluted sequentially with stepwise solvent systems (200 mL each) of dichloromethane, dichloromethane/chloroform/ethyl acetate mixture (8:10:10; 4:3:3, v/v/v), and ethyl acetate, respectively. The corresponding four column chromatography fractions, F1–F4 were obtained. Solvents in each fraction were collected and removed by using *Buchi RE 111* rotary evaporator.

2.2.4. Chromatographic isolation of antioxidant compounds

Thin layer chromatography was used for the preliminary investigations of the fractions, determination of suitable solvent systems for subsequent separation of the compounds using column chromatography, checking the purity of the compounds isolated as well as confirmation of their identity. Throughout this work, pre-coated TLC plates of silica gel-60 F_{250}, 5.0–6.5 cm length, 0.2 mm thickness, coated on aluminium foil (Merck), were used unless stated otherwise. The solvent mixture (200 mL) of diethyl ether and hexane (2:1, v/v) was submitted to a silica gel column (1.5 cm internal diameter. × 80 cm) which had been prepared by pouring a slurry of silica gel and solvent mixture into a glass chromatographic tube (1.5 cm internal diameter × 15 cm) and left overnight. Two millilitres of fraction 1 (approximately 4.26 g) were then applied on to a silica gel (40 g, 230–400 mesh, Fluka) column and eluted sequentially with stepwise solvent systems (50 mL each) of hexane, hexane/diethyl ether mixture (9:1; 6:1; 3:1, v/v), and diethyl ether, respectively. The eluate was collected in 2 mL fractions. The fractions were examined by TLC, and those with the same profile (Fractions 1–4, 5–29, 30–57, 58–66, and 67–70) were combined to yield the corresponding five subfractions F1a–F1e, respectively. Solvent in each subfraction was removed under a stream of nitrogen gas.

F1b (34.75%) was chromatographed on a silica gel column with diethyl ether/hexane mixture (2:1, v/v), affording 32 fractions. TLC analysis was performed using chloroform/ethyl acetate/dichloromethane (4:5:6, v/v/v) as the mobile phase. Fraction 4–28 were further purified by silica gel column with chloroform/ethyl acetate (8:2, v/v) as mobile phase, to yield 1040 mg of compound **1**. F1c (38.9%) was purified with silica gel and eluted with diethyl ether/hexane mixture (2:1, v/v), affording 35 fractions. Fraction 4–31 were chromatograhped over a silica gel column and eluted with chloroform/ethyl acetate (8:2, v/v), affording 625 mg of compound **1** and 152 mg of compound **2**. F1d (12.9%) on further purification with diethyl ether/hexane mixture (4:1, v/v) on a silica gel column, yielded 38 mg of compound **3**. F1a and F1e which contained no significant amount of polyphenols as indicated by the TLC analysis, were not purified further.

2.2.5. Qualitative analysis

2.2.5.1. Gas chromatography mass spectrometry (GC/MS). The three isolated compounds and CPAE II were analyzed qualitatively by gas chromatography and mass spectrometry (GC/MS), using a Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph, model 6890 series, equipped with a MSD 5973, and a Hewlett-Packard Vectra Pentium computer. A fused silica capillary column, 30 m long, 0.25 mm internal diameter, 0.25 μm thickness was used. The column initial temperature was programmed at 40 °C (1 min) with an increase of 5 °C/min up to 230 °C (10 min). Helium was used as the carrier gas. The temperature for injector and detector were 250 °C. An injection volume of 0.2 μL was used.

2.2.5.2. Nuclear magnetic resonance spectroscopy. NMR spectra were recorded using either a Bruker Avance 300 spectrometer or a Bruker Avance 400 spectrometer (Bruker Biospin, Rheinstetten, Germany), operated at 300 MHz and 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, respectively. The isolated compounds were prepared in deuterated solvents in 5 mm NMR tubes (Sharp, Gosney, & Rowley, 1989). The deuterated solvent used was chloroform-*d* and the chemical shifts were measured relative to the TMS signal. All the experiments were carried out at room temperature.

2.3. Methods

2.3.1. Radical scavenging activity (RSA)

The free radical scavenging activity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method proposed by Blois (1958). Briefly, 100 μL of sample at different concentration (0.20–0.10 mg/mL) were added to 3 mL of DPPH solution (9.25 mg in 250 mL). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity, and vice versa. The EC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, was calculated from the results and used for comparison. The capability to scavenging the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging effect } (\%) = [(A1 - A2/A1) \times 100]$$

where $A1$ is the absorbance of the control (DPPH solution without test sample) and $A2$ is the absorbance in the presence of the test sample. BHA, BHT, alpha-tocopherol and ascorbic acid were used as reference compounds.

2.3.2. Ferric reducing antioxidant power (FRAP)

The antioxidant capacity was determined using a modification of the FRAP assay described by Langley-Evans (2000). The FRAP reagent was prepared from 300 mM, pH 3.6, acetate buffer, 20 mM ferric chloride and 10 mM 2,4,6-tripyridyl-*S*-triazine made up in 40 mM hydrochloric acid. All three solutions were mixed together in the ratio of 25: 2.5: 2.5 (v/v/v). The FRAP assay was performed using reagents preheated to 38 °C. Prior to analysis, the initial absorbance of 3 mL of the reagents, and a 3 mL acetate buffer used as blank, were measured at 593 nm. The samples (100 μL) were transferred into the cuvettes containing the reagent and the mixtures were shaken thoroughly for 15 s. The mixtures in cuvettes were examined after 90 min using a UV–vis spectrophotometer and the absorbance values at 593 nm were recorded. The results reported are mean values expressed as milligrams of ascorbic acid equivalents per gram sample (AEAC).

2.3.3. Phosphomolybdenum assay

The antioxidant activity of samples were evaluated by the phosphomolybdenum method according to the procedure of Prieto, Pineda, and Aguilar (1999). An aliquot of 0.1 mL of sample solution (1 mM in dimethyl sulphoxide) was combined in a 4 mL vial with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. The results reported are mean values expressed as milligrams of ascorbic acid equivalents per gram sample (AEAC).

2.3.4. ABTS radical cation assay

ABTS (2,2′-azinobis-(3-ethylbenzothiazoneline-6-sulphonic acid)) was used as the free radical provider and was generated by reacting this compound (7.4 mM) with potassium persulphate (2.45 mM) overnight (Re et al., 1999). It is applicable for both hydrophilic and lipophilic compounds (Mathew & Abraham, 2004). DPPH radical is a free and neutral radical that is acquired directly without preparation while ABTS radical cation must be generated by enzymatic or chemical reactions (Brandwilliams, Cuvelier, & Berset, 1995; Miller & Rice-Evas, 1997). The solution was diluted to obtain an absorbance of between 1.5 and 2.5 at 414 nm with 98% of ethanol, before used. Reagents (3 mL) were transferred to the glass cuvettes with one of them containing 3 mL ethanol as blank. The initial absorbance of the reagents in the glass cuvettes at 414 nm was recorded. The samples (10–50 μL, 1.0 mg/mL) were transferred into the cuvettes containing the reagent and the mixtures were shaken thoroughly. The mixture in the cuvettes was examined after 90 min using a UV–vis spectrophotometer. The capability to scavenging the ABTS radical cation was calculated using the following equation:

$$\text{ABTS radical cation scavenging capability } (\%) = [(A1 - A2/A1) \times 100]$$

where $A1$ is the absorbance of the control (ABTS solution without test sample) and $A2$ is the absorbance in the presence of the test sample.

Also, the antioxidant capacity of the extract was obtained by comparing the change of absorbance at 414 in a test reaction mixture containing extracts with that containing Trolox. The results reported are mean values expressed as milligrams of Trolox equivalents per gram sample (TEAC).

2.4. Statistical analysis

All determination of antioxidant capacity by DPPH radical, phosphomolybdenum, FRAP, and ABTS radical cation assays are conducted in triplicates. The values expressed are the mean of three measurements.

3. Result and discussion

3.1. Extraction and fractionation

Several methods have been used for separating and obtaining polyphenol rich fractions (Achladas, 1991), with solvent extraction and silica gel column chromatography being the most common (Boocock, Kallury, & Tidwell, 1983; Schabron, Hurtubise, & Silver, 1979). Solvent extraction is more frequently used for isolation of antioxidant and both extraction yield and activity of extracts are strongly dependant on the solvent, due to the different antioxidant potential of compounds with different polarity (Julkunen-Tiito, 1985; Marinova & Yanishlicha, 1997).

Table 1
Chemical composition of the polyphenols extract of pyroligneous acid, *Rhizophora apiculata* (CPAE II)

No.	Compounds	RT (min)	Composition (%)	Mode of identification
	Alcohols (0.34 %)			
1	1-Chloro-2-propanol	8.55	0.103	GC–MS, AS
2	(*S*)-(+)-2-Chloro-1-propanol	9.82	0.010	GC–MS
3	1,2-Propanediol	14.86	0.116	GC–MS, AS
4	2-(2-Hydroxypropoxy)-1-propanol	22.61	0.107	GC–MS
	Ketones (5.65 %)			
5	3-Methyl-2-cyclopenten-1-one	13.40	0.311	GC–MS, AS
6	2,3-Dimethyl-2-cyclopenten-1-one	13.91	0.106	GC–MS, AS
7	3-Ethyl-2-hydroxy-2-cyclopenten-1-one	20.04	0.217	GC–MS
8	2-Hydroxy-3-methyl-2-cyclopenten-1-one	20.84	4.697	GC–MS, AS
9	2-Ethyl-2-methyl-1,3-cyclopentanedione	21.76	0.213	GC–MS, AS
10	2,4-Dimethyl-3-heptanone	29.68	0.105	GC–MS
	Furan and pyran derivatives (16.48 %)			
11	Tetrahydro-2-furanmethanol	12.96	0.203	GC–MS
12	2-Furaldehyde	15.48	1.857	GC–MS, AS
13	Dihydro-5-methyl-2-(3H)-furanone	15.68	0.312	GC–MS, AS
14	Dihyro-2-(3H)-furanone	16.11	0.116	GC–MS
15	2,5-Dihydro-3,5-dimethyl-2-furanone	16.63	0.218	GC–MS
16	5-Methyl-2-(5H)-furanone	17.34	0.204	GC–MS
17	3-Methyl-2-(5H)-furanone	18.21	0.249	GC–MS
18	Dihydro-4,4-dimethyl-2,3-furandione	18.66	0.131	GC–MS
19	4-Methyl-5H-furan-2-one	21.98	0.113	GC–MS, AS
20	3-Hydroxy-2,6-dimethyl-4H-pyran-4-one	23.12	0.413	GC–MS
21	3-Hydroxy-2-methyl-4H-pyran-4-one	23.84	12.450	GC–MS, AS
22	2H-1-Benzopyran-2-one	32.33	0.217	GC–MS
	Guaicol and derivatives (6.63 %)			
23	2-Methoxyphenol (guaicol)	21.48	0.304	GC–MS, AS
24	4-Methyl-2-methoxyphenol (4-methylguaicol)	23.49	0.319	GC–MS
25	4-Ethyl-2-methoxyphenol (4-ethylguaicol)	28.55	0.415	GC–MS, AS
26	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	34.27	3.040	GC–MS, AS
27	4-Hydroxy-3-methoxyacetophenone (acetovanillone)	35.45	0.318	GC–MS, AS
28	4-(2-Propenyl)-2-methoxyphenol (eugenol)	36.56	1.023	GC–MS, AS
29	4-Hydroxyacetophenone	39.02	0.211	GC–MS
	Phenol and derivatives (5.91 %)			
30	Phenol	24.54	3.767	GC–MS, AS
31	4-Methylphenol (*p*- cresol)	26.02	1.196	GC–MS, AS
32	2-Methylphenol	26.16	0.111	GC–MS, AS
33	4-Ethylphenol	27.75	0.219	GC–MS, AS
34	2-Hydroxy-6-methylbenzaldehyde	30.43	0.112	GC–MS
35	4-Methoxyphenol (mequinol)	31.68	0.073	GC–MS
36	4-Hydroxy-3-methylacetophenone	37.19	0.218	GC–MS, AS
37	3-Hydroxybenzaldehyde	37.65	0.216	GC–MS, AS
	Syringol and derivatives (50.43 %)			
38	2,6-Dimethoxyphenol (syringol)	29.57	49.340	GC–MS, AS
39	3,4-Dimethoxyphenol	33.19	0.413	GC–MS, AS
40	2,6-Dimethoxy-4-(2-propenyl)-phenol	33.97	0.317	GC–MS
41	4-Hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde)	40.09	0.225	GC–MS
42	4-Hydroxy-3,5-dimethoxyacetophenone	40.98	0.136	GC–MS
	Pyrocatechol (8.74 %)			
43	1,2-Benzenediol (catechol)	30.88	5.374	GC–MS, AS
44	3-Methoxycatechol	32.16	3.053	GC–MS, AS
45	1,4-Benzenediol (hydroquinone)	41.76	0.317	GC–MS, AS
	Alkyl aryl ether (0.49 %)			
46	Trimethoxybenzene	28.19	0.218	GC–MS, AS
47	1,2,4-Trimethoxybenzene	30.16	0.106	GC–MS
48	3,4,5-Trimethoxytoluene	31.84	0.162	GC–MS

(*continued on next page*)

Table 1 (*continued*)

No.	Compounds	RT (min)	Composition (%)	Mode of identification
	Nitrogenated compounds (0.53 %)			
49	*N*,*N*-Dimethylacetamide	10.85	0.318	GC–MS, AS
50	1-Methyl-2-pyrolidinone	17.23	0.214	GC–MS
	Carbohydrate derivatives (0.22 %)			
51	1,4:3,6-Dianhydro-α-D-glucopyranose	27.23	0.217	GC–MS
	Lignin dimers (0.05 %)			
52	Diphenylacetaldehyde	32.98	0.151	GC–MS

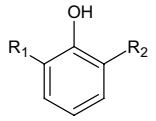
RT: retention time; GC–MS: identification by comparison of mass spectra to those in the library, NIST (National Institute of Standards and Technology); AS: identification by comparison of their mass spectra of authentic standards.

Ethyl acetate has been used for extraction of low molecular weight phenols from oak wood (Fernandez de Simon, Cadahia, Conde, & Garcia-Vallejo, 1996). Using the acid base extraction method, 250 mL of the CPAE (1.020 g/mL) were further extracted and successfully yielded 20 mL of CPAE II (1.175 g/mL). The polyphenols content in the pyroligneous acid of *R. apiculata* was determined to be approximately 2.98%. The composition of CPAE II was analyzed by gas chromatography–mass spectroscopy (GC/MS). Compounds were identified by their retention times, by their mass spectra, by comparing their mass spectra with those in a commercial library (National Institute of Standards and Technology (NIST)), and in some cases, using authentic standards. Out of 57 peaks, 52 compounds were identified, representing 95.47% of the total polyphenols (Table 1). The study indicates that CPAE II contains a high number of components arising, basically from wood carbohydrate thermal degradation such as ketones (5.65%), alcohols (0.34%), furan and pyran derivatives (16.48%) and some others. In addition, it contains a significant number of components arising from lignin thermal degradation such as phenol and derivatives (5.91%), guaiacol and derivatives (6.63%), syringol and derivatives (50.43%), pyrocatechol and derivatives (8.74%), as well as a trace amounts of nitrogenated components (0.53%), carbohydrate derivatives (0.22%) and lignin dimmers (0.05%). Four column chromatographic fractions (F1–F4) were obtained from CPAE II by means of thin layer chromatography and silica gel column chromatography. The yields of fraction 1, 2, 3 and 4 were 36.23%, 46.81%, 5.42% and 10.34%, respectively. In addition, CPAE II was found to possess superior antioxidative activities when evaluated with some classical assays including DPPH radical scavenging activity, phosphomolybdenum assay and ferric reducing antioxidant power (FRAP). CPAE II exhibited superior free radical scavenging activity with EC_{50} value = 0.1045 mg/mL, which was approximately 2.3-fold smaller than that of ascorbic acid (EC_{50} value = 0.2562 ± 0.0023 mg/mL). Results also showed that CPAE II had high molybdenum (VI) reducing power and ferric reducing power. Values were 5512 ± 294 and 5112 ± 342 mg ascorbic acid equivalents/g of the sample, respectively. Moderately high ferric reducing power of approximately 715 ± 42 mg ascorbic acid equivalents/g of F1 was found. It showed strong free radical scavenging activity with EC_{50} value = 0.1336 ± 0.0007 mg/mL, which was approximately 2-fold smaller than that of ascorbic acid and BHA.

3.2. Isolation and identification of compounds

Silica gel column chromatography of Fraction 1 yielded five subfractions (F1a–F1e). The yields of F1a–F1e were 6.52, 34.75, 38.90, 12.90 and 4.93%, respectively. TLC analysis and repeated silica gel column chromatography of F1b, F1c, and F1d gave three antioxidant compounds. The chemical structure confirmation of the isolated compounds was accomplished by comparing the mass spectra obtained to those in a commercial library (National Institute of Standards and Technology (NIST)). Similarly, ^{1}H and ^{13}C NMR data were obtained at spectral to those published. Compound **1** (1665 mg) was isolated from F1b and F1c as yellowish to light brown crystal, melting point range: 53–56 °C. Its molecular weight was 154 Da, determined by mass spectrum. Compound **2** (152 mg) was isolated from F1c as white solid, its molecular weight was 110 Da, melting point range: 104–106 °C. Compound **3** (38 mg) was isolated from F1d as light brownish crystals. Its molecular weight was 140 Da; melting point range: 40–43 °C. The results showed that F1 was dominated by compound **1** (39.08%), compound **2** (4.21%) and compound **3** (1.01%). The structure of compound **1** was determined by spectral analysis to be syringol (2,6-dimethoxyphenol). MS of compound **1** showed M^+ at *m*/*z* 154 which corresponds to the molecular formula $C_8H_{10}O_3$. IR (ZnSe, cm^{-1}): 3489 (O—H) cm^{-1}, 1615, 1480 (C=C aromatic), 1214 (C—O). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.89 (s, 6H, —CH_3), 6.58 (d, 2H, Ar), 6.79 (dd, 1H, Ar).^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 56.3 (—CH_3), 104.9, 119.1, 147.4 (Ar), 134.9 (—C—OH). Compound **1** showed identical R_f with syringol standard, 0.68 and 0.78 when the solvent system was chloroform/ethyl acetate/dichloromethane (4:5:6, v/v/v) and dichloromethane/ethyl acetate (8:2, v/v), respectively. The structure of compound **2** was determined by spectral analysis to be catechol (1,2-dihydroxybenzene). MS of compound **2** showed M^+ at *m*/*z* 110 which corresponds to the molecular formula $C_6H_6O_2$. IR (ZnSe, cm^{-1}): 3450 (O—H) cm^{-1}, 1619, 1470 (C=C aromatic), 1256 (C—O).^{1}H NMR

(400 MHz, $CDCl_3$): δ (ppm) 6.80 (d, 2H, Ar), 6.83 (d, 2H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 115.5, 121.3 (Ar), 143.5 (—C—OH). Compound **2** showed identical R_f with catechol standard, 0.89 and 0.74 when the solvent system was acetone/methanol (3:1, v/v) and chloroform/dichloromethane/ethyl acetate (4:5:6, v/v/v), respectively. The structure of compound **3** was determined by spectral analysis to be 3-methoxycatechol (1,2-dihydroxy-3-methoxybenzene). MS of compound **3** showed M^+ at *m/z* 140 which corresponds to the molecular formula $C_7H_8O_3$. IR (ZnSe, cm^{-1}): 3412 (O—H) cm^{-1}, 1618, 1481 (C=C aromatic), 1208 (C—O).^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.90 (s, 3H, —CH_3), 5.35 (d, 1H, —OH), 5.44 (d, 1H, —OH), 6.48 (d, 1H, Ar), 6.61 (d, 1H, Ar), 6.76 (dd, 1H, Ar).^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 56.7 (—CH_3), 104.0, 109.5, 120.3 (Ar), 133.1 (—(CH_3O)—C—C—OH), 144.6 (—C—OH), 147.8 (—C—CH_3). Compound **3** showed identical R_f with 3-methoxycatechol standard, 0.76 and 0.82 when the solvent system was acetone/methanol (3:1, v/v) and chloroform/dichloromethane/ethyl acetate (4:5:6, v/v/v), respectively. The detailed MS, ^1H and ^{13}C NMR data of compound **1**, **2** and **3** are listed in Table 2. The identity of the compounds was further confirmed by GC co-injection with authentic standards. Compound **1, 2** and **3** were identified as syringol, catechol and 3-methoxycatechol, respectively (Fig. 1).



Compound 1, $R_1 = OCH_3$, $R_2 = OCH_3$
Compound 2, $R_1 = H$, $R_2 = OH$
Compound 3, $R_1 = OH$, $R_2 = OCH_3$

Fig. 1. Chemical structures of the compounds isolated from the pyroligneous acid, *Rhizophora apiculata*. Compound **1**, $R_1 = OCH_3$, $R_2 = OCH_3$; compound **2**, $R_1 = H$, $R_2 = OH$; compound **3**, $R_1 = OH$, $R_2 = OCH_3$.

3.3. Free radical scavenging activity

Relative stable DPPH has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Jao & Ko, 2002). The reduction capacity of DPPH radicals was determined by the decrease in its absorbance at 517 nm (Duh, 1998). The isolated compounds of *R. apiculata* showed a concentration-dependant antiradical activity by reducing the stable radical DPPH to a yellowish coloured diphenylpicrylhydrazine derivative (Table 3). Two traditional food antioxidants ascorbic acid and alpha-tocopherol; together with two synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as controls. The quality of the antioxidants in the extracts was determined by the EC_{50} values and percentage of radical scavenging activity shown in Table 3. A low EC_{50} value or a high percentage of radical scavenging activity indicates strong antioxidant activity in a sample tested. Among the three isolated compounds, the most potent radical scavenger was catechol (EC_{50} of 0.1239 ± 0.0004 mg/mL), followed by 3-methoxycatechol ($EC_{50} = 0.2001 \pm 0.0005$ mg/mL) and finally syringol ($EC_{50} = 0.2218 \pm 0.0009$ mg/mL) as the least active compound. Catechol and 3-methoxycatechol with di-ortho phenolic structure showed higher scavenging power than the mono-phenolic compound, syringol. An increase in the number of hydroxyl groups in the phenyl

Table 3
Percentage of free radical scavenging activity and EC_{50} values of syringol, catechol, 3-methoxycatechol and the reference compounds. Values are means of three independent determinations ± SD

Compounds[a]	Free radical scavenging activity (%)	EC_{50} (mg/mL)
Ascorbic acid	39.04 ± 0.36	0.2562 ± 0.0023
BHA	37.63 ± 0.53	0.2657 ± 0.0038
Alpha-tocopherol	20.96 ± 0.44	0.4765 ± 0.0097
BHT	18.95 ± 0.53	0.5272 ± 0.0148
Syringol	45.09 ± 0.18	0.2218 ± 0.0009
Catechol	80.71 ± 0.26	0.1239 ± 0.0004
3-Methoxycatechol	51.05 ± 0.18	0.2001 ± 0.0005

[a] The test samples were prepared at 0.10 mL/mL.

Table 2
Spectral and melting point data of the compounds isolated from column chromatography fraction (F1) from the polyphenols extract of pyroligneous acid, *Rhizophora apiculata*

Compound	^1H NMR (400 Hz)[a]	^{13}C NMR (100 Hz)[a]	IR (cm^{-1}) (KBr)	MS (*m/z*)	Melting point (°C)
1	3.89 (s,6H),6.58 (d,2H),6.79 (dd,1H)	56.3, 104.9, 119.1, 134.9, 147.4	1214 (C—O), 1368 (CH_3), 1458 (C—H), 1615, 1480 (C=C aromatic), 3489 (O—H)	51, 65, 68, 79, 93, 111, 139, 154[b]	53–56
2	6.80 (d,2H), 6.83 (d,2H)	115.5, 121.3, 143.5	1256 (C—O), 1619, 1470 (C=C aromatic), 3450 (O—H)	51, 53, 63, 64, 81, 82, 110[b]	104–106
3	3.90 (s,3H), 5.35 (d,1H), 5.44 (d,1H), 6.48 (d,1H), 6.61 (d,1H), 6.76 (dd,1H)	56.7, 104.0, 109.5, 120.3, 133.1, 144.6, 147.8	1208 (C—O), 1354 (CH_3), 1618, 1481 (C=C aromatic), 3412 (O—H)	38, 39, 51, 53, 68, 79, 97, 107, 125, 140[b]	40–43

[a] TMS as internal standard.
[b] Molecular ions.

ring increased the radical scavenging activity, due to the fact that more hydrogen atoms of the phenolic hydroxyl groups can be donated to stabilize the free radicals (Rice-Evas et al., 1996; Shahidi & Wanasundara, 1992). Also notable was that the presence of an additional methoxy group to a dihydroxybenzene might eliminate its free radical scavenging activity, as reported by Bortolomeazzi, Sebasitianutto, Toniolo, and Plzzariellow (2007). Also, it is important to note that all three antioxidant compounds isolated showed a higher scavenging capacity than ascorbic acid, BHT, BHA and alpha-tocopherol.

3.4. Ferric reducing antioxidant power assay

This simple and reliable test measures the reducing potential of an antioxidant reacting with a ferric 2,4,6 – tripyridyl-*S*-triazine [Fe(III)–TPTZ] complex and producing a coloured ferrous 2,4,6-tripyridyl-*S*-triazine [Fe(II)–TPTZ] complex by a reductant at low pH, was adopted. This complex has an intense blue colour that can be monitored at 593 nm. The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Hadas, 1995). A higher absorbance indicates a higher ferric reducing power. Catechol showed the highest ferric reducing power, followed by 3-methoxycatechol, and the lowest was syringol. Values were 2283 ± 168, 1560 ± 155 and 635 ± 35 mg ascorbic acid equivalents/g of the sample, respectively (Table 4). In this assay, dihydroxybenzenes with di-ortho phenolic structure showed higher reducing power than syringol. The result and structure-reducing capacity relationship were similar to that of DPPH radical scavenging capacity which has been discussed previously. In brief, the reducing power of three isolated compounds exhibited the descending order of: catechol > 3-methoxycatechol > syringol.

3.5. Evaluation by phosphomolybdenum assay

The phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of green phosphate/Mo(V) complex with the maximal absorption at 695 nm. The assay was successfully used to quantify vitamin E in seeds (Prieto et al., 1999) and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant polyphenols. A higher absorbance indicates a higher antioxidative activity. 3-Methoxycatechol showed the greatest antioxidative efficacy, followed by catechol and syringol. Values were 2396 ± 194, 1861 ± 95 and 1556 ± 86 mg ascorbic acid equivalents/g of the sample, respectively (Table 4). In the ranking of the antioxidant capacity obtained by this method, 3-methoxycatechol showed an antioxidant capacity higher than that of catechol, a behaviour opposite to that in DPPH radical and FRAP assays. The result indicates that an extra methoxy group in the di-ortho phenolic structure of 3-methoxycatechol actually increased the electron donating ability and, therefore, increased the reducing capability of the compound. It was reported that antioxidants show lacks antioxidant activity in the DPPH radical assay might demonstrate a very strong antioxidant activity in the phosphomolybdenum assay (Marwah, Fatope, Al Mahrooqi, Varma, & Al Burtamani, 2007). Hydrogen and electron transfer from antioxidants to DPPH radical and Mo(VI) complex occur in the DPPH radical and phosphomolybdenum assays, respectively. The transfers occur at different redox potentials in the two assays and also depend on the structure of the antioxidant. An increase in the number of both hydroxyl and methoxy groups in the phenyl ring increases the reducing ability of dihydroxybenzenes and dimethoxyphenols. In brief, the antioxidative ability of the isolated compounds and standards decreased in order of 3-methoxycatechol > catechol > syringol.

3.6. ABTS radical cation assay

The ABTS radical cation scavenging capacities of each compounds and Trolox were examined. The three antioxidant compounds differed in their ABTS radical cation scavenging capacities. 3-Methoxycatechol exhibited stronger scavenging effect ($90.82 \pm 6.33\%$) compared to that of catechol ($89.39 \pm 6.86\%$), suggesting that the presence of —OCH3 ortho to the OH group may significantly enhance the ABTS radical cation scavenging of dihydroxybenzenes. Among the three isolated compounds, syringol with only one hydroxyl group was the least active ABTS radical cation scavenger ($83.98 \pm 4.71\%$). The TEAC values for 3-methoxycatechol, catechol and syringol at the maximum

Table 4
Ferric reducing antioxidant power (FRAP), molybdenum (VI) reducing power (phosphomolybdenum assay) and ABTS cation radical scavenging activity of syringol, catechol and 3-methoxycatechol. Values are means of three independent determinations ± SD

Extracts	Antioxidant activity			
	$AEAC_{FRAP}$ (mg AA/g)[a]	$AEAC_{Phosphomolybdenum}$ (mg AA/g)[a]	$TEAC_{ABTS}$ (mg TR/g)[b]	ABTS radical cation Scavenging activity (%)
Syringol	635 ± 35	1556 ± 86	956 ± 40	83.98 ± 4.71
Catechol	2283 ± 168	1861 ± 95	1022 ± 53	89.39 ± 6.86
3-Methoxycatechol	1560 ± 155	2396 ± 194	1039 ± 51	90.82 ± 6.33

[a] Milligrams of ascorbic acid equivalents per gram sample.
[b] Milligrams of Trolox equivalents per gram sample.

concentration studied were found to be 1039 ± 51, 1022 ± 53 and 956 ± 40 mg equivalents/g of the sample, respectively (Table 4). The trend in antioxidant activity was similar to that of phosphomolybdenum assay, indicating that an increase in the number of both hydroxyl and methoxyl groups in the phenyl ring increases the ABTS radical cation scavenging activity of dihydroxybenzenes and dimethoxyphenols.

4. Conclusions

The three antioxidant compounds present in pyroligneous acid of *R. apiculata* significantly differed in their abilities to reduce ferric and molybdenum(VI) ion, as well as to scavenge DPPH radicals and ABTS radical cations. Both hydroxyl and methoxy substituents influenced the antioxidant properties of dihydroxybenzenes and dimethoxyphenols. The trend in antioxidant capacity was similar in DPPH radical and ferric reducing assays, with catechol > 3-methoxycatechol > syringol. However, the trend in phosphomolybdenum and ABTS radical cation assay was 3-methoxyphenol > catechol > syringol. In addition, syringol which is present in relatively high percentage appears to be the main compound responsible for the antioxidant activity of Fraction 1. This is the first report on the isolation and identification of these three antioxidant compounds from pyroligneous acid of *R. apiculata*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2007.09.044.

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