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Antioxidant and radical scavenging activities of the pyroligneous acid from a mangrove plant, *Rhizophora apiculata*

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Abstract

Total phenolics content, free radical scavenging activity, reducing power, and antioxidant activity of the pyroligenous acid from a mangrove plant, *Rhizophora apiculata* were evaluated. Dichloromethane extraction of the raw pyroligneous acid successfully yield 2 extracts, i.e. concentrated pyroligneous acid (CPA) and concentrated pyroligneous acid extract (CPAE). Phenolic contents in CPAE and CPA, expressed as (±)-catechin equivalents/g of the sample were 5465 ± 367 mg and 2502 ± 152 mg, and expressed as gallic acid equivalents/g of the sample were 2919 ± 209 mg and 1348 ± 90 mg, respectively. CPAE exhibited superior free radical scavenging activity with EC_{50} value = 0.1235 mg/ml, or 80.96% of free radical scavenging capability. The ferric reducing power of CPAE was approximately 3.7, 5.1, 6.1, and 21.3 times higher than that of ascorbic acid, BHA, BHT and alpha-tocopherol. In phosphomolybdenum assay, CPAE showed the greatest antioxidant efficacy ($A_{695} = 1.278$) compared to those of CPA and different standards. In addition, the free radical scavenging activity, ferric reducing power and total antioxidative activity of CPAE and CPA showed positive correlation with their total phenolic content with R^2 values ranging from 0.9624 to 0.9979.
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Keywords: *Rhizophora apiculata*; Pyroligneous acid; Total phenolic content; DPPH radical scavenging; Antioxidant assay; Ferric reducing antioxidant power

1. Introduction

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. Pyroligneous acid was first developed in Japan when scientists and researchers discovered that wood vinegar from some trees has powerful detoxifying abilities. This has been confirmed in Japan after hundred of experiments with oak, sakura and beech tree sap extracts (Japanese white oak contains the highest concentration of pyroligneous acid). In India, mangrove plant has been used in folklore medicine for several diseases (Kirtikar & Basu, 1935). It was reported that thef alkaline extract from leaves of a mangrove plant, *Rhizophora apiculata* species successfully inhibited the HIV replication and HIV-induced cytopathic effects (Premanathan et al., 1999).

In Malaysia, pyroligneous acid of *R. apiculata* species has been used for ages as sterilizing agent, deodorizer, fertilizer, antimicrobial and growth promoting agent. To the best of our knowledge, however, little information is available about the antioxidant properties of pyroligneous acid of *R. apiculata* species.

Nowadays, antioxidants have gained more importance because of their positive involvement as health promoters in conditions such as cardiovascular problems, atherosclerosis, treatment of many forms of cancer, and the ageing process (Packer, 1999). Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical scavengers (Duh, 1998; Yen & Duh, 1994). In

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recent years, interest has considerably increased in finding naturally occurring antioxidants for use in food or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Ito, Fukushima, Hasegawa, Shibata, & Ogiso, 1983; Zheng & Wang, 2001). A number of synthetic antioxidants, such as 2- and 3-*tert*-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA), and *tert*-butylhydroquinone (TBHQ) have been added to foodstuffs but because of toxicity issues, their use is being questioned (Valentao et al., 2002). Therefore, attention has been directed towards the development and isolation of natural antioxidants from plant sources. Crude extracts of spices, herbs, and other plant materials rich in polyphenolics are increasingly of interest to the food industry because they have the capacity to retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004).

The aim of the this study was to evaluate the total phenolics content, radical scavenging activity, reducing power and antioxidant activity of pyroligneous acid of *R. apiculata*, by using of a number of classical assays, and to assess whether this species could be sources of natural antioxidant for pharmaceutical and food application.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), alpha-tocopherol, ascorbic acid, Folin Ciocalteu's reagent, dimethyl sulphoxide, Whatman No. 1 filter paper, pottasium ferricyanide, ferric chloride, sodium carbonate, ACS grade methanol and all analytical grade chemicals were purchased from Sigma (Sigma Aldrich GmbH, Sternheim, Germany).

2.2. Plant materials and extraction

2.2.1. Plant material

The raw pyroligneous acid form *R. apiculata* was obtained from Kuala Sepetang Charcoal village in Perak, Malaysia. The obtained raw pyroligneous acid has a clear reddish brown colour which is similar to the pleasing hue of black tea, beer or wine. The sample was stored in the dark at below 20 °C before use.

2.2.2. Preparation of concentrated pyroligneous acid (CPA)

A sample of raw pyroligneous acid was filtered through Whatman No. 1 filter paper to eliminate solid particles and dust. The filtrate obtained was then concentrated to a 10:1 volume ratio using a Buchi RE 111 rotary evaporator at 80 °C. The product, after water elimination, was a thicker brown liquid, or concentrated pyroligneous acid (CPA).

Preliminary experiments were carried out prior to the evaporation process, to determine the total phenolic content and antioxidative activity of the raw pyroligenous acid. No significant difference was observed (<0.05%) with regards to the total phenolic content and antioxidative activity between the raw pyroligneous acid and the CPA prepared at different temperatures during water elimination (Table 1). As the raw pyroligneous acid is the water condensate of the vapors produced during wood carbonization, temperature between 80 °C and 100 °C is suggested to effectively eliminate the water content when there is no other alternative such as vacuum evaporator.

2.2.3. Dichloromethane solvent extraction

The 25 ml of CPA obtained was then exhaustively extracted with 25 ml of dichloromethane using a separatory funnel. Dichloromethane is used for experimental purpose, but is not a recommended solvent for extraction to be used by the industry. Two phases could be distinguished on standing after 30 min. The organic layer, the fraction soluble in dichloromethane, or CPAE was then collected. This step was repeated five times. The aqueous fraction, after water elimination by evaporation, gave rise to Residue I.

2.3. Methods

2.3.1. Determination of total phenolic content by Folin Ciocalteau assay

The total phenolics content in pyroligneous acid preparation was estimated by a colorimetric assay based on the procedure described by Swain and Hillis (1959) and Naczk

Table 1
Comparison between the total phenolic content of raw pyroligneous acid and concentrated pyroligneous acid prepared at different temperature

Assay	Raw pyroligneous acid[a]	Concentrated pyroligneous acid[b]			
		40 °C	60 °C	80 °C	100 °C
mg Gallic acid equivalents	1320 ± 64	1324 ± 58	1330 ± 60	1356 ± 62	1348 ± 90
mg (±)-Catechin equivalents	2470 ± 108	2460 ± 124	2548 ± 108	2538 ± 106	2502 ± 152
EC_{50} (mg/ml)	0.2246	0.2228	0.2304	0.2288	0.2303
A_{695} (nm)	0.574	0.568	0.582	0.594	0.553
A_{593} (nm)	0.798	0.784	0.812	0.805	0.816

[a] All the determination of total phenolic content and antioxidative capability were carried out at room temperature.
[b] Concentrated pyroligneous acid prepared at different temperature during the process of water elimination were tested for their total phenolic content and antioxidative capability.

and Shahidi (1989) with slight modifications. A 1 ml aliquot of sample was pipetted into a test tube containing 8 ml of methanol. After mixing the contents, 5 ml of the 10 % Folin Ciocalteu's reagent and 4 ml of the 0.75% sodium carbonate solution were added. The contents were vortexed for 15 s and then left to stand at room temperature for 30 min. Absorbance measurements were recorded at 765 nm and (±)-catechin and gallic acid were used in the construction of curves. Estimation of the phenolic compound was carried out in triplicate. The results reported are mean values expressed as mg of (±)-catechin and gallic acid equivalents per gram of sample.

2.3.2. Radical scavenging activity (RSA)

The free radical scavenging activity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method proposed by Blois (1958). A 100 μl of sample at different concentration (0.20–0.10 mg/ml) was 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 scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scavenging Effect } (\%) = [(A_1 - A_2/A_1) \times 100]$$

where A_1 is the absorbance of the control reaction and A_2 is the absorbance in the presence of the sample. BHA, BHT, alpha-tocopherol and ascorbic acid were used as controls. The experiment was carried out in triplicate and the results are mean values.

2.3.3. 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. A higher absorbance indicates a higher ferric reducing power.

2.3.4. Evaluation of antioxidant activity

The antioxidant activity of pyroligneous acid was 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 sulfoxide) was combined in a 4 ml vial with 1 ml of reagent solution (0.6 M sulfuric 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. BHA, BHT, alpha-tocopherol and ascorbic acid were used as controls. The experiment was carried out in triplicate and the results are mean values.

3. Result and discussion

3.1. Determination of total phenolic content

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups (Hatano, Edamatsu, Mori, Fujita, & Yasuhara, 1989). The phenolic content may contribute directly to the antioxidative action (Duh, Tu, & Yen, 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka, Kuei, Nagashima, & Taguchi, 1998). Most of the work dealing with phenolic content in natural products use gallic acid and (±)-catechin as standards (independently of phenolic species detected), the content of phenolic in this work was expressed as gallic acid equivalents and (±)-catechin equivalents to facilitate comparison (Jerez, Pinelo, Sineiro, & Nunez, 2004). As shown in Table 2, the total phenolic content increased with increased concentration, ranging from 0.02 to 0.10 mg/ml, for both pyroligneous acid extracts. At 0.10 mg/ml, the total phenolic content of CPAE was approximately 5465 ± 367 mg (±)-catechin or 2919 ± 209 mg gallic acid equivalents/g of the sample, which was about two times higher compared to CPA. The total phenolic content of CPA was approximately 2502 ± 152 mg (±)-catechin or 1348 ± 90 mg gallic acid equivalents/g of the sample. In short, the results suggest

Table 2
Milligram (±)-catechin and gallic acid equivalent of phenols of different concentration of CPA, CPAE

Sample	Concentration (mg/ml)	mg equivalent (±)-Catechin per gram sample	mg equivalent Gallic acid per gram sample
CPA	0.02	518 ± 34	296 ± 27
	0.04	1062 ± 71	585 ± 60
	0.06	1546 ± 93	842 ± 73
	0.08	2017 ± 130	1091 ± 94
	0.10	2502 ± 152	1348 ± 90
CPAE	0.02	1441 ± 85	786 ± 59
	0.04	2781 ± 160	1496 ± 64
	0.06	3800 ± 264	2037 ± 142
	0.08	4756 ± 303	2543 ± 251
	0.10	5465 ± 367	2919 ± 209

Each value is expressed as mean ± S.D.

that both extracts of pyroligneous acid are a rich source of polyphenolic compounds.

3.2. Free radical scavenging activity

DPPH· is a stable free radical and accepts electron or hydrogen radical to become a stable diamagnetic molecule (Soares, Dins, Cunha, & Ameida, 1997). A freshly prepared DPPH· solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH· (by providing hydrogen atom or by electron donation) and convert it to a colourless product, resulting in a decrease in absorbance at 517 nm (Yamaguchi, Takamura, Matoba, & Terao, 1998). In brief, the reduction capacity of DPPH· was determined by the decrease in its absorbance at 517 nm, which is reduced by antioxidants (Duh et al., 1999). Fig. 1 illustrates a significant decrease in the concentration of DPPH· due to the scavenging ability of the pyroligneous acid extracts and standards. We used ascorbic acid, BHA, BHT, and alpha-tocopherol as standards. As shown in Fig. 2, both pyroligneous acid extracts and standards showed increased free radical scavenging activity with the increased concentration. The quality of the antioxidants in the extracts was determined by the EC_{50} values shown in Table 3. A low EC_{50} value indicates strong antioxidant activity in a sample. The EC_{50} value of the CPAE was 0.1235 mg/ml, being the lowest indicating that this extract exhibited the highest radical scavenging effect. The EC_{50} values of CPA was 0.2303 mg/ml, which was about 2-fold smaller than alpha-tocopherol. BHT has the lowest radical scavenging activity, with the EC_{50} value (0.5278 mg/ml).The scavenging effect on the DPPH radical

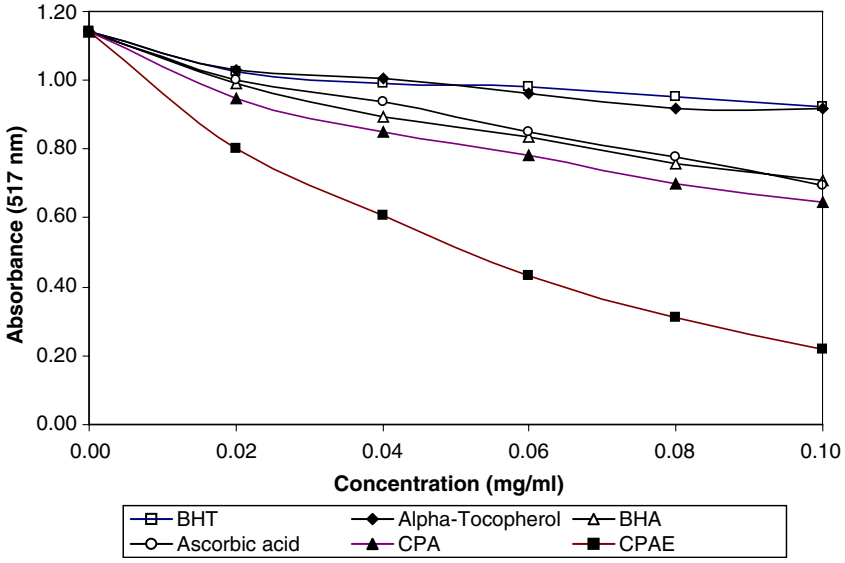

Fig. 1. Free radical scavenging activity of CPA, CPAE, BHT, BHA, alpha-tocopherol, and ascorbic acid.

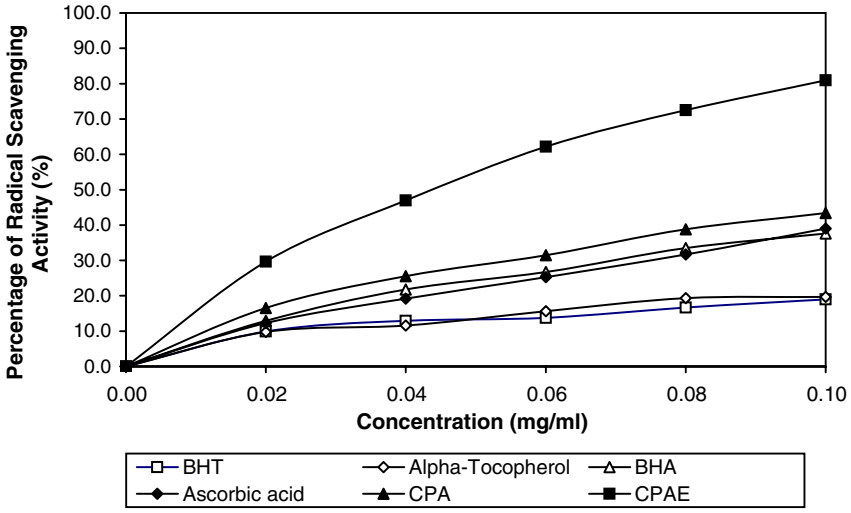

Fig. 2. Percentage of free radical scavenging activity of CPA, CPAE, BHT, BHA, alpha-tocopherol, and ascorbic acid.

Table 3
Free radical scavenging activity of the CPA, CPAE and standards

Extracts/standards	EC_{50} (mg/ml)	Free radical scavenging activity (%)
Ascorbic acid	0.2562	39.04
BHA	0.2657	37.63
Alpha-tocopherol	0.4771	21.01
BHT	0.5278	18.95
CPA	0.2303	43.42
CPAE	0.1235	80.96

decreased in that order: CPAE > CPA > ascorbic acid > BHA > alpha-tocopherol > BHT. The result indicates that both CPAE and CPA have significant effects on scavenging free radicals.

3.3. Ferric reducing/antioxidant power (FRAP)

Ferric reducing antioxidant assay, a simple and reliable test that depends upon the reduction of ferric 2,4,6–tripyridyl-*S*-triazine [Fe(III)–TPTZ] to the 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. Although initially elaborated for estimation of the total antioxidant activity in biological samples, this method was then modified for routine analysis of the antioxidative activity of pure chemical substances and plant extracts (Niemeyer & Metzler, 2003 ; Tsai, McIntosh, Pearce, Camden, & Jordan, 2002).

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. As displayed in Fig. 3, both pyroligneous acid extracts and standards showed increased ferric reducing power with the increased concentration. At 0.10 mg/ml, the reducing power of CPAE ($A_{593} = 2.263$) was far superior to that of CPA ($A_{593} = 0.816$), ascorbic acid ($A_{593} = 0.609$), BHA ($A_{593} = 0.372$), BHT ($A_{593} = 0.443$), and alpha-tocopherol ($A_{593} = 0.106$). In fact, CPAE was approximately 3.7, 5.1, 6.1, and 21.3 times stronger than of ascorbic acid, BHA, BHT and alpha-tocopherol. The ferric reducing power of CPAE at 0.02 mg/ml ($A_{593} = 0.379$) was comparable to that of BHA at a concentration of 0.10 mg/ml ($A_{593} = 0.372$). The reducing power of CPA ranged from 0.02 to 0.10 mg/ml ($A_{593} = 0.135$–0.816) was less effective than CPAE but, much better than BHA, BHT, alpha-tocopherol and ascorbic acid. The ferric reducing power of CPA at 0.10 mg/ml ($A_{593} = 0.816$) was comparable to CPAE ($A_{593} = 0.831$). Ascorbic acid showed the highest ferric reducing power among the standards used but, less effective reducing power to that of CPA and CPAE At concentration ranging from 0.02 to 0.10 mg/ml; alpha-tocopherol showed the lowest reducing power and, with only approximately 4.7% and 13.0% of the reducing power of CPAE and CPA, respectively, at 0.10 mg/ml. In brief, the reducing power of pyroligneous extracts and standards exhibited the descending order of: CPAE > CPA > ascorbic acid > BHT > BHA > alpha-tocopherol.

3.4. Evaluation of antioxidant activity

The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by an antioxidant and the formation of green phosphate/Mo (V) complex with 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. As displayed in Fig. 4, all extracts and standards showed increasing antioxidant activity with increased concentration and, CPAE

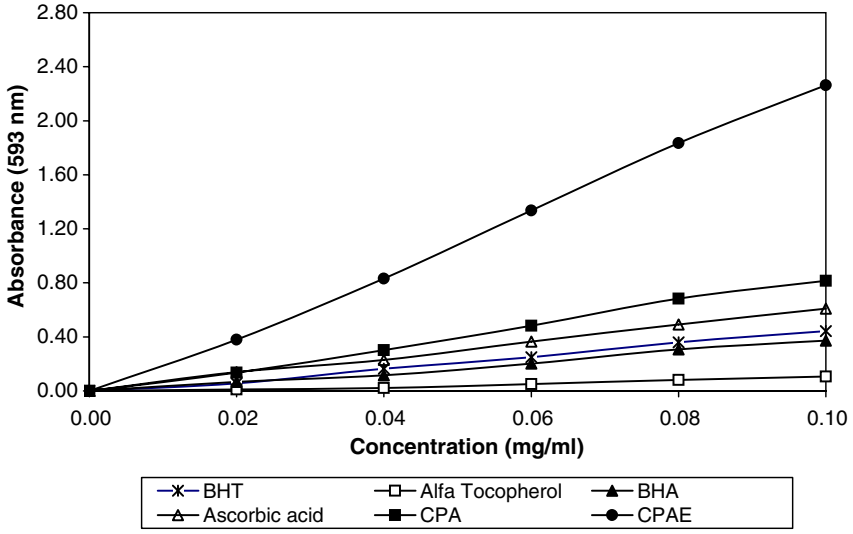

Fig. 3. Ferric reducing power of CPA, CPAE, ascorbic acid, BHT, BHA, and alpha-tocopherol at different concentration.

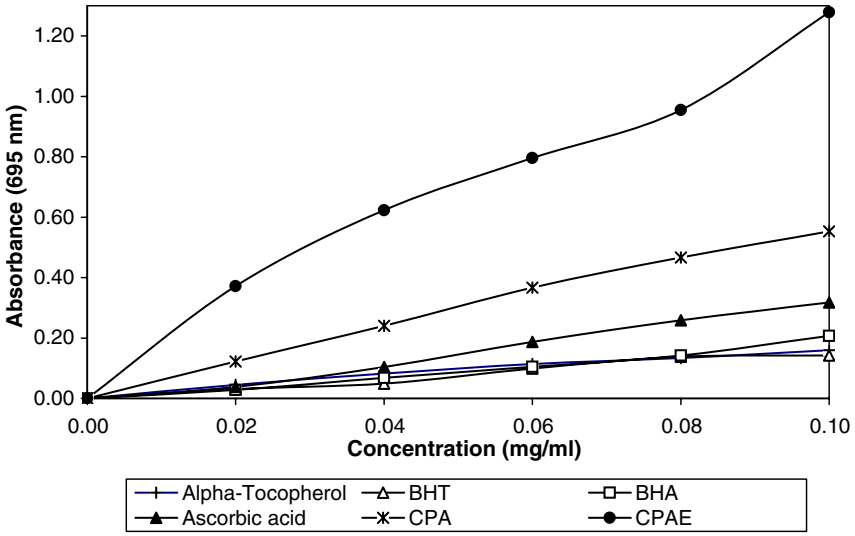

Fig. 4. Antioxidative activity of CPA, CPAE, ascorbic acid, BHT, BHA, and alpha-tocopherol at different concentration.

showed the greatest antioxidative efficacy ($A_{695} = 1.278$) compared to those of CPA and standards used. As can seen from Fig. 4, the antioxidative activity of CPAE increased exponentially ranging from 0.02 to 0.10 mg/ml. At 0.02 mg/ml concentration, CPAE showed comparable antioxidative activities to that of 0.06 mg/ml concentration of CPA. The antioxidative activity of CPA at 0.06 mg/ml ($A_{695} = 0.367$) was higher than that of ascorbic acid ($A_{695} = 0.318$), BHA ($A_{695} = 0.207$), alpha-tocopherol ($A_{695} = 0.160$) and BHT ($A_{695} = 0.142$) at 0.10 mg/ml. At concentration of 0.40 and 0.10 mg/ml, the antioxidant activity of ascorbic acid ($A_{695} = 0.099$, 0.219) was comparable to that of 0.02 and 0.04 mg/ml concentration of CPAE ($A_{695} = 0.109$, 0.217). Ascorbic acid exhibited better antioxidative activity than alpha-tocopherol, BHT and BHA at concentrations ranging from 0.02 to 0.10 mg/ml. The result indicated that the antioxidative ability of the extracts and standards decreased in order of CPAE > CPA > ascorbic acid > BHA > alpha-tocopherol > BHT.

3.5. Correlations between total phenolic content and antioxidative function

Figs. 5 and 6 show that the free radical scavenging activity, reducing power and antioxidative activity of CPAE and CPA correlated well with their total phenolic content ($R^2 = 0.9624–0.9979$). CPAE which exhibited the highest antioxidative activity, radical scavenging activity and ferric reducing power has the highest amount of phenolic content. The results indicate that polyphenolic compounds are the major antioxidants in pyroligneous acid of *R. apiculata.*

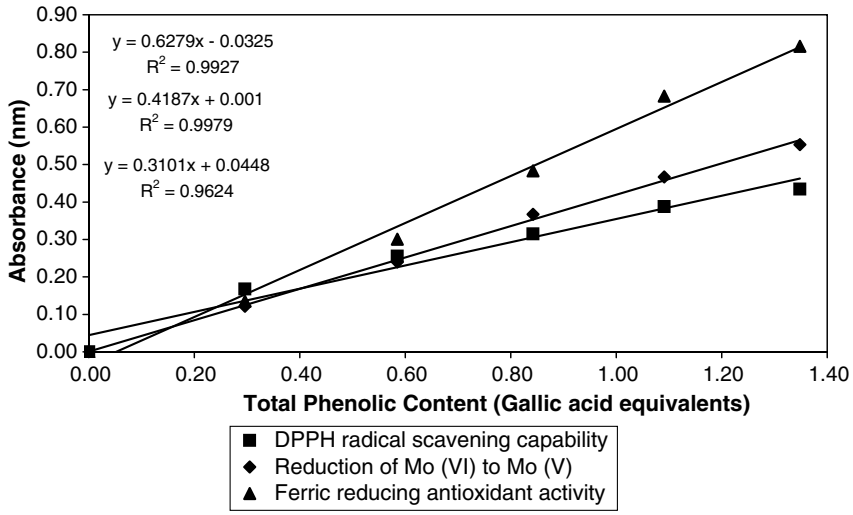

Fig. 5. Correlation between the total phenolic content and the antioxidant activities of CPA. There is a linear positive correlation, between total phenolic content and the antioxidative activity ($R^2 = 0.9979$) , total phenolic content and ferric reducing power ($R^2 = 0.9927$), total phenolic content and the free radical scavenging activit ($R^2 = 0.9624$), respectively.

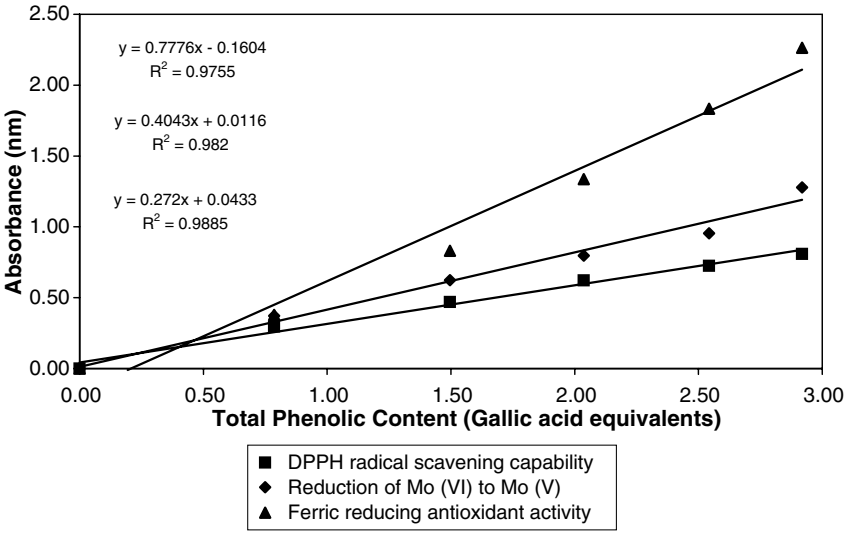

Fig. 6. Correlation between the total phenolic content and the antioxidant activities of CPAE. There is a linear positive correlation, between total phenolic content and the antioxidative activity ($R^2 = 0.9820$), total phenolic content and ferric reducing power ($R^2 = 0.9755$), total phenolic content and the free radical scavenging activit ($R^2 = 0.9885$), respectively.

4. Conclusions

Pyroligneous acid of *R. apiculata* species is a rich source of antioxidants. Pyroligneous acid or wood vinegar contains many beneficial ingredients such as organic acetic acid, methanol, ketones, aldehydes and polyphenols. The dichloromethane extracts of pyroligneous acid, CPAE evidently showed higher total phenolics content, more effective antioxidative activity, reducing power, and DPPH radical scavenging activity when compared to different standards such as BHA, BHT, alpha-tocopherol, and ascorbic acid. In addition, the raw pyroligneous acid, CPA was also a potent antioxidant since it has high phenolic content and showed comparable efficacy with BHA, BHT, alpha-tocopherol and ascorbic acid in the antioxidant assays. Also, it showed that polyphenolic compounds are the major antioxidants in pyroligneous acid of *R. apiculata.* In the present work, however, the components responsible for the antioxidant activities are unclear. Therefore, further work is in progress for the isolation and identification of the antioxidant components in pyroligneous acid of *R. apiculata*.

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