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Research Article

RADICAL SCAVENGING ACTIVITY OF LIGNIN EXTRACTED FROM OIL PALM EMPTY FRUIT BUNCH AND ITS EFFECT ON GLUTATHIONE-S-TRANSFERASE ENZYMES ACTIVITY

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ABSTRACT

Objective: Lignin consists of cell wall phenylpropanoid polymers widespread in all vascular plants and is present in lignocellulosic waste such as oil palm empty fruit bunch (OPEFB). The presence of valuable compounds in OPEFB lignin such as vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde promise additional value to lignin especially in pharmaceutical and food supplement industries.

Methods: In this study, lignin was isolated from OPEFB using three types of extraction techniques (soda, kraft, and organosolv)and characterized via calorimetric, chromatographic, and spectroscopic methods. The antioxidant activity of all three types of OPEFB lignin by 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical-scavenging effect was examined. OPEFB lignin extracts were therefore further investigated for their influence on glutathione-S- transferase enzymes activity in rat liver cytosolic fractions using spectrophotometer assay.

Results: The kraft OPEFB lignin showed the highest DPPH free radical scavenging effect compared to the other. High-performance liquid chromatography analysis revealed that all OPEFB lignin were mainly formed by *p*-hydroxyphenyl (H) guaiacyl (G) and syringyl (S) units whereas vanillin and syringaldehyde were the main compounds in soda, kraft, and organosolv OPEFB lignin. Soda OPEFB lignin showed the most effective inhibition of rat liver cytosolic glutathione S-transferases activity toward 1-chloro-2,4-dinitrobenzene followed by kraft OPEFB lignin.

Conclusion: This study found that the antioxidant activities of all three types of OPEFB lignin were related to the phenolic hydroxyl group (ArOH) contents and their M_w, M_n, and M_w/M_n values. The effect of OPEFB lignin on the inhibition of GST enzymes activity shows good correlation with the total flavonoid content of each OPEFB lignin.

Keywords: Oil palm empty fruit bunch lignin, Antioxidant activity, Glutathione-S-transferase, Syringaldehyde, Vanillin.

INTRODUCTION

Malaysia is one of the leading agricultural countries in the world with exports including palm oil, cocoa and rubber. Simultaneously, agricultural activities also generate a substantial amount of lignocellulosic agriculture wastes. As far as oil palm is concerned, Malaysia has approximately 362 palm oil mills, processing about 82 million tons of fresh fruit bunch and producing an estimated 33 million tons of crop residue annually in the form of empty fruit bunch (EFB), fibers, trunks, fronds, and shells [1]. As the main exporter of palm oil in the world, utilization of oil palm biomass for the production of environmental-friendly biomaterials has become an attractive approach instead of creating pollution problems [2]. Furthermore, the development of lignocellulosic industry is predicted to be an economy booster for small scale farmers.

Oil palm EFB (OPEFB) contained polymeric component such as hemicellulose (20-21%), cellulose (35-40%), and lignin (17-21%) [3]. Lignin is considered as a natural phenolic polymer which is one of the most abundant natural polymer composing of up to one-third of the material found in plant cell walls. Lignin is one of the co-products that is produced from the chemical pulping process in pulp and paper industry. Different types of lignin can be obtained from different pulping process. For example, soda lignin can be obtained from sodium pulping process, kraft lignin from kraft pulping process while organosolv lignin can be obtained from ethanol pulping process.

Some recent studies also revealed the efficacy of lignin from different sources such as sugar cane, hybrid poplar, and wood as antioxidants as well as medicine and dietary products [4-10]. Lignin is a free radical scavenger. It stabilizes the reactions induced by oxygen and its radical species [11,12]. As a major component in dietary fiber, lignin has an ability to inhibit the activity of enzymes related to the generation of superoxide anion radicals and to obstruct the growth and viability of cancer cells [6]. Therefore, lignin has promising applications in cosmetic, pharmaceutical, and food industries. Previous study [13] had reported that eight compounds were found in degraded soda OPEFB lignin. These are vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, syringic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and ferulic acid. The concentrations of all eight compounds in all three types of OPEFB lignin were also determined quantitatively.

The quantitative determination of all compounds in OPEFB lignin is important to add more information of all three types of OPEFB lignin beside their other physical characterization. The presence of valuable compounds in OPEFB lignin such as vanillin and syringaldehyde also promised an additional value to lignin especially in pharmaceutical and food supplement area. Before OPEFB lignin can be applied in the pharmaceutical and food supplement development area, the possibility of it interacting with other drugs or pharmaceutical agents exists. The investigation of potential drug interaction with OPEFB lignin via inhibition of drug metabolizing enzymes such as glutathione-S- transferase (GST) activity is needed to ensure product safety.

GST activity is present in most animal tissues. GST utilizes glutathione (GSH) to scavenge electrophilic xenobiotics as part of an organism's defense mechanism against the mutagenic, carcinogenic, and toxic effects of such compounds. This is so because, GST enzymes account for the defense against oxidative stress and they detoxify endogenous harmful compounds such as the breakdown products of lipid peroxidation or DNA hydroperoxides [14,15].

In this study, the effects of lignin (soda, kraft, and organosolv) extracted from OPEFB on GST enzymes activity were investigated along with their

antioxidant capacities. In addition, characterizations of lignin extracted from oil palm empty fruit bunch from three different extraction methods were conducted.

METHODS

Materials

The raw material used in this study consisted of oil palm empty fruit bunch long fiber supplied by Sabutek (M) Sdn. Bhd., Teluk Intan, Malaysia, a local company specializing in recycling of oil palm lignocellulosic wastes. The EFB fiber was pulped in a 10 L stainless steel rotary digester unit together with 20% w/v NaOH (cooking liquor) for 3 hrs at a maximum cooking temperature of 170°C. The mixture of cooking liquor to EFB fiber was in the ratio of 2:8 (v/w). Prior to the pulping process, the fiber was soaked in water for 2 days to remove dirt.

Soda, kraft, and organosolv pulping process

For kraft pulping, the EFB fiber was pulped in a 10 L stainless steel rotary digester unit together with 19% of active alkali and a 25% of sulfidity with water to fiber ratio of 8. The digester was heated from room temperature to 170°C for 1 hr and continued at 170°C for 2 hrs. For soda pulping, 25% of active alkali with no percentage of sulfidity was applied and similar conditions were used. For organosolv pulping process, a 1.0 L glass-lined pressure Parr reactor with a Parr 4842 temperature controller (Parr Instrument Company, Moline, IL) was employed for the pretreatment. About 22.0 g of EFB (dry mass) was treated with ethanol/water mixture in a volume ratio of 65:35 with sulfuric acid as a catalyst [16]. The solid to liquid ratio used was 1:8. The reactor was heated at room temperature to 190°C for 1 hr and continued at 190°C for 45 minutes.

Preparation of OPEFB soda, kraft, and organosolv lignin

The soda and kraft lignin were precipitated from concentrated black liquor by acidifying them to pH 2.0 using a calculated amount of 20% (v/v) sulfuric acid. Both precipitates were filtered and washed with distilled water at pH 2.0, which was prepared using the same acid as in the earlier step. The soda and kraft lignin were dried in an oven at 55°C for 24 hrs [17]. The preparation of organosolv OPEFB lignin was carried out by washing the pre-treated EFB with warm ethanol/water (8:1, 3 × 50.0 mL). The washes were combined and 3 volumes of water were added to precipitate the ethanol organosolv lignin, which was collected by centrifugation and air dried.

Purification of OPEFB lignin

The purification of lignin sample respectively was conducted by extracting in the soxhlet apparatus for 6 hrs with n-pentane to remove wax and lipids. The precipitate was filtered and washed with pH 2 water to remove the excess n-pentane and non-lignin phenolic compounds which may remain in the black liquor. The purified lignin was then dried further in the oven at 45°C for another 24 hrs before storage in plastic bottles for further analysis.

Characterization of OPEFB lignin

All OPEFB lignin samples were analyzed using spectroscopic, chromatographic, and calorimetric techniques. The ^{31}P nuclear magnetic resonance (NMR) spectra were acquired after derivatizing 25 mg of lignin samples respectively with 2-chloro-4,4,5,5-tetramethyl-1-1,3,2- dioxaphospholane (TMDP)[18]. Cyclohexanol was used as an internal standard. Quantitative NMR spectra were acquired using an inverse-gated decoupling (Waltz-16) pulse sequence with a 30° pulse angle and 25 seconds pulse day. The ^{31}P NMR data were processed offline using XW in NMR processing software.

The number average molecular weight (M_n) and weight average molecular weight (M_w) of lignin were determined by gel permeation chromatography (GPC) after acetylation of lignin to allow dissolution in dimethylformamide (DMF). In brief, 20 mg of lignin was dissolved in a 1:1 acetic anhydrate/pyridine mixture (1.00 mL) and stirred for 24 hrs at room temperature. Ethanol (25.0 mL) was then added to the reaction mixture, left for 30 minutes, and subsequently removed with rotary evaporator. The addition and removal of ethanol was repeated several times to ensure complete removal of acetic acid and pyridine from the sample. Following that, the acetylated OPEFB lignin samples were dissolved in chloroform (2.0 mL) and added drop-wise to diethyl ether (100 mL) followed by centrifugation. The precipitate were washed three times with diethyl ether and dried under vacuum (762 mm of Hg) at 40°C for 24 hrs. GPC analysis for OPEFB lignin samples were performed using a Hewlett-Packard 1090 series high-performance liquid chromatography (HPLC) system consisting of an auto sampler, a UV detector and three columns of styragel HR1, HR3, and HR4 (Waters Inc.) linked in series using DMF as the eluent. The derivatized lignin was dissolved in DMF (1 mg/mL) and the solution was filtered through a 0.45 µm filter. Then the filtered solution (20 µL) was injected into the HPLC system and was detected using the UV detector at 280 nm. Standard polystyrene samples were used to construct a calibration curve. Data were collected with Agilent ChemStation rev. A.10.01 and analyzed with Agilent GPC Addon rev. A.02.02 software.

The total flavonoid content of OPEFB lignin was determined using a modified colorimetric method by Zhishen *et al.* [19]. An aliquot of OPEFB lignin was mixed with 1.25 mL distilled water respectively. The mixture was mixed with 75 µL of 5% sodium nitrite solution. After standing for 6 minutes, the mixture was combined with 1500 µL of 10% aluminium chloride. About 0.5 mL of 1M sodium hydroxide and 275 µL of distilled water were added after 5 minutes later. The absorbance of the solution at 510 nm was then measured using spectrophotometer (PerkinElmer Lambda 45). A calibration curve using quercetin in a concentration range of 0.02-0.64 mg/mL was prepared. The total flavonoid content of oil palm EFB lignin were expressed as quercetin equivalent (QE), which reflected the flavonoid content as amount of quercetin in OPEFB lignin sample. All experiment was performed in triplicates.

Antioxidant activity

The free radical scavenging activity of OPEFB lignin was measured in terms of hydrogen donating or radical scavenging ability using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH)[20]. 1 mg/mL of all samples were dissolved in methanol. All samples were prepared from different concentrations (0.002-0.8 mg/mL) and 200 µL of sample from each concentration was loaded into 96 well plate. To this mixture, 50 µL of DPPH solution was added into each of the well plate. After 30 minutes incubation at room temperature (22-24°C) in a dark place, the absorbance was measured at 517 nm by using spectrophotometer (PerkinElmer Lambda 45) against methanol as the blank. A control contained 1 mL methanol and 2 mL 0.1 mM DPPH methanol. Free radical scavenging activity (RSA) of the oil palm EFB lignin and single compounds were determined according to the following formula:

$$\text{Free RSA (\%)} = (A_c - A_s)/A_c \times 100$$

Where A_s is the absorbance of DPPH and sample, A_c is the absorbance of control.

Nitrobenzene oxidation process

Nitrobenzene oxidation was carried out by adding 50 mg of each dry OPEFB lignin sample into a mixture of 7 mL of 2 M NaOH and 4 mL of nitrobenzene in a 15 mL steel autoclave. The autoclave was sealed tightly with a screw cap fitted with a Teflon gasket and heated to 165°C for 3 hrs in a preheated thermostatic oil bath. After the heating period, the autoclave was cooled with ice water. The mixture was then transferred to a liquid-liquid extractor for continuous extraction with chloroform (5-20 mL) to remove any nitrobenzene reduction product and excess of nitrobenzene. The oxidation mixture was acidified with concentrated HCl to pH 3-4 and further extracted with chloroform (5-15 mL). The solvent from the second chloroform solution was removed by using a rotary evaporator at 40°C under reduced pressure to obtain the nitrobenzene oxidation mixture. The mixture was then dissolved into dichloromethane and made up to

10 mL. This mixture was used as a stock solution for further analysis of the oxidation products [21].

HPLC analysis

HPLC was used to quantify the nitrobenzene mixture of OPEFB lignin. A 0.2 mL of stock solution was pipetted into a 25 mL volumetric flask and made up with acetonitrile-water (1:2 v/v). The resulting sample solution was filtered through a Millipore membrane (pore size 0.45 µm) to remove high molecular weight contaminant. About 10 µL of the filtrate was injected into a HPLC system (Shimadzu) equipped with Hypersil bond C18 column (particle size 5 µm, 25 mm × 4.6 mm i.d.) to qualitatively and quantitatively determine the oxidation products. A mixture of acetonitrile: water (1:8) containing 1% acetic acid was used as an eluent with a flow rate of 1 mL/ min. The eluent was monitored with a UV detector at 280 nm [21].

Animals

Male Sprague Dawley rats (150-200 g) were obtained from the Animal House of Universiti Sains Malaysia (USM). The rats were maintained under controlled temperature (25±2°C), 12 hrs light/12 hrs dark conditions for 1 week before the start of the experiments. They were provided with water and food *ad libitium*. Animals were maintained and handled according to the recommendations of the USM Ethical Committee which approved the design of the experiments. Six rats were obtained and remain untreated. The rats were sacrificed by cervical dislocation and the livers were taken out for microsomes and cytosolic fraction preparation.

Preparation of rat liver cytosolic fraction

Cytosolic liver fractions are prepared as described by Gibson and Skett [22]. Rat livers were removed immediately after sacrifice was rinsed with ice-cooled distilled water followed by ice-cooled 67 mM potassium phosphate buffer (pH 7.4), blotted dry, and weighted. Isolated rats liver samples were homogenized in 3 volumes of 67 mM potassium phosphate buffer (pH 7.4) containing 1.15% potassium chloride, using a Potter-Elvehjem homogenizer. After centrifugation of the homogenate fraction at 12,500× g for 20 minutes at 4°C, the resultant supernatant was decanted to ultracentrifuge tubes (Optiseal™) and centrifuge at 100,000 × g for 60 minutes in a Optima™ TLX refrigerated ultracentrifuge (Beckman Coulter, Inc., USA). The supernatant contains microsomal and soluble cytosolic fractions. The supernatants are kept at −80°C until use. Protein concentration of the cytosolic fractions was determined by Lowry's method using bovine serum albumin as the standard [23].

GST enzymes activity assay

OPEFB lignin (soda, kraft, and organosolv)(0.01-500 µg/ml) were used for *in vitro* study. Total GST activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate [22]. The reaction mixture contained potassium phosphate buffer (100 mM; pH 6.5), 0.01 mL of GSH (30 mM), and equal volume of soda, kraft, and organosolv oil palm EFB lignin. The reaction was initiated by adding 10 mg/mL of cytosolic fractions. The final volume is 0.3 mL. Control petri dishes contained all of the above except soda, kraft, organosolv OPEFB lignin, extracts and was replaced with distilled water. The reaction was monitored by microplate spectrophotometer at 340 nm (Powerwave X-340®; Biotek).

Statistical analysis

GST enzyme activity was compared to the respective control group; and means and standard deviations were calculated. Analysis was carried out using Dunnet Test. The levels of significance were set at $p<0.05$.

RESULTS

^{31}P NMR analysis of soda, kraft, and organosolv OPEFB lignin

Fig. 1 shows the ^{31}P NMR spectrum of soda, kraft, and organosolv OPEFB lignin while the characteristics of each OPEFB lignin calculated from^{31}P NMR data were tabulated in Table 1. Fig. 1, along with Table 1; show that all 3 types of lignin were mainly formed by guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units which consist of phenolic hydroxyl group (ArOH) content. From a previous study [10], the RSA value is positively correlated to phenolic hydroxyl group (ArOH) content, consistently with other studies [9,11,24].

Molecular weight distribution of soda, kraft, and organosolv OPEFB lignin

Further, the weight average (M_w), number average (M_n), molecular weight and polydispersity (M_w/M_n) of all three OPEFB lignin extracts were investigated using GPC and their M_w, M_n, and M_w/M_n values were tabulated in Table 2. The M_w and M_n of all three types of OPEFB lignin increased in the order of soda (M_w = 2930 g/mol; M_n = 1484 g/ mol)> organosolv (M_w = 1675 g/mol; M_n = 1098 g/mol)> kraft (M_w = 1628 g/ mol; M_n = 815 g/mol).

Total flavonoid content

Table 2 demonstrates the total flavonoid content in OPEFB lignin stated as mg quercetin equivalent/g of lignin. The total flavonoid of soda OPEFB lignin showed the highest value (491.31±7.79 mg QE/g of lignin) followed by kraft (245.65s±0.81 mg QE/g of lignin) and organosolv OPEFB lignin (240.93±4.80 mg QE/g of lignin).

RSA of soda, kraft, and organosolv OPEFB lignin

Table 2 shows the calculated half-inhibition concentration (IC_{50}), which is the effective concentration of the three types of OPEFB

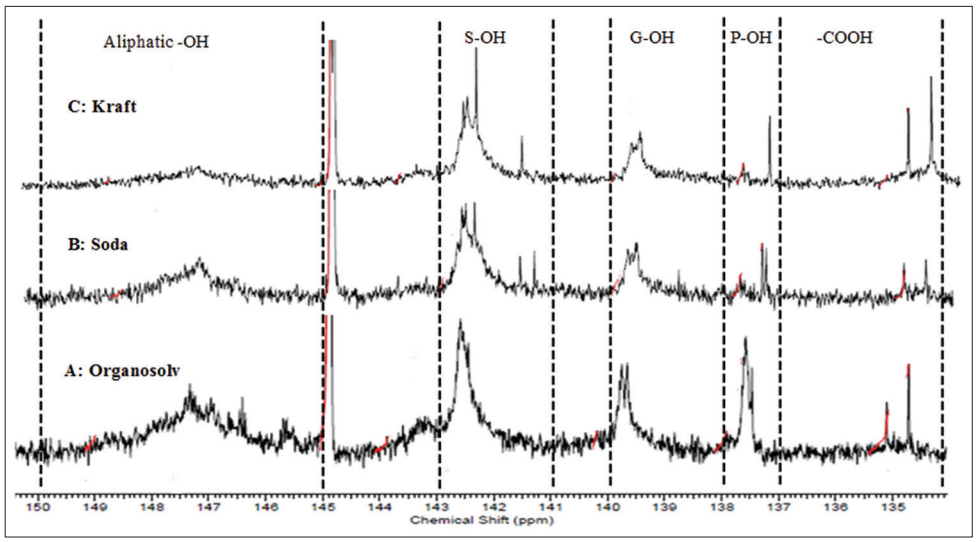

Fig. 1: Quantitative 31P nuclear magnetic resonance spectra of A: Organosolv B: Soda and C: Kraft oil palm empty fruit bunch lignin

lignin required to decrease initial DPPH concentration to 50%. These IC_{50} values were obtained by interpolation from the linear regression analyses of data shown in Fig. 2. As shown in Fig. 2 and Table 2, the kraft OPEFB lignin showed the lowest IC_{50} value (0.056±0.002 mg/mL) compared to organosolv and soda OPEFB lignin (0.094±0.004 mg/mL and 0.237±0.005 mg/mL, respectively).

HPLC analyses of soda, kraft, and organosolv OPEFB lignin

The compounds present in OPEFB lignin were analyzed via HPLC. Fig. 3 shows the HPLC chromatogram of soda, kraft, and organosolv OPEFB lignin. The compounds obtained and the percentage (w/w) of each compound was summarized in Table 3. From Table 3, vanillin (3.334±0.042% in soda; 2.553±0.037% in kraft and 2.840±0.143% in organosolv) and syringaldehyde (7.074±0.126% in soda; 5.109±0.007% in kraft and 5.803±0.281% in organosolv) were found to be the predominant compounds followed by *p*-hydroxybenzaldehyde (0.283±0.004% in soda; 0.353±0.006% in kraft, and 0.551±0.022% in organosolv).

Effects of OPEFB lignin on GST enzymes activity

The effects of OPEFB lignin on the activity of GST enzymes activity were assessed by measuring the conjugation activity with CDNB (Fig. 4). The percent inhibition of GST enzymes activity was evaluated in the presence of varied oil palm EFB lignin concentrations (0.001- 100 µg/ mL). Soda, kraft, and organosolv OPEFB lignin showed considerable inhibition on the GST activity, but the IC_{50} value of organosolv OPEFB lignin cannot been determined due to the percentage of inhibition being <50%. Comparing soda and kraft OPEFB lignin for their IC_{50} values, soda OPEFB lignin was the most effective inhibitor of cytosolic GST activity toward CDNB, with the value IC_{50} of 18.27±1.28 µg/mL. Whereas, kraft OPEFB lignin showed an IC_{50} value of 23.20±2.15 µg/ mL (Table 2).

DISCUSSION

GST enzymes play an important role in multidrug resistance to chemotherapy. In this study, lignin extracted from OPEFB (soda, kraft, and organosolv) were screened for their effects on GST activity besides

Table 1: Lignin extracted from OPEFB characteristics calculated from ^{31}P NMR data

Chemical shift range δ^{31}P-NMR	Assignment	Soda OPEFB[a] (mmol/g)[b]	Kraft OPEFB[a] (mmol/g)[b]	Organosolv OPEFB[a] (mmol/g)[b]
134-135	Carboxylic acid	0.0146	0.0751	0.0149
137-138	*p*-hydroyphenyl OH (H)	0.0155	0.0225	0.0431
138-140	Guaiacyl OH (G)	0.0639	0.1056	0.0693
141-143	Syringyl OH (S)	0.1572	0.2556	0.1675
~145	Cyclohexanol (IS)			
145-150	Aliphatic OH	0.1083	0.8746	0.1598
Total phenolic content (S+G+H)		0.2366	0.3837	0.2799

[a]Lignin samples phosphorylated with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane, [b]Determination by integration with cyclohexanol as an internal standard, NMR: Nuclear magnetic resonance, OPEFB: Oil palm empty fruit bunch

Table 2: Comparison of 50% inhibition values of DPPH radical scavenging, GST activity with the total flavonoid content in OPEFB lignin

OPEFB lignin	DPPH scavenging IC_{50} (mg/mL)	GST enzymes activity IC_{50} (µg/mL)	M_w (g/mol)	M_n (g/mol)	M_w/M_n	TF (mg QE/g of lignin)
Ascorbic acid	0.002	NA	NA	NA	NA	NA
Soda	0.237±0.005	18.27±1.28	2930	1484	1.97	491.31±7.79
Kraft	0.056±0.002	23.20±2.15	1628	815	2.00	245.65±0.81
Organosolv	0.094±0.004	ND	1675	1098	1.53	240.93±4.80

SD: Standard deviation, Results are expressed as mean±SD, DPPH: 2,2-diphenyl-1-picrylhydrazyl, OPEFB: Oil palm empty fruit bunch, GST: Glutathione-S-transferase, ND: Not detected, QE: Quercetin equivalent, NA: Not available

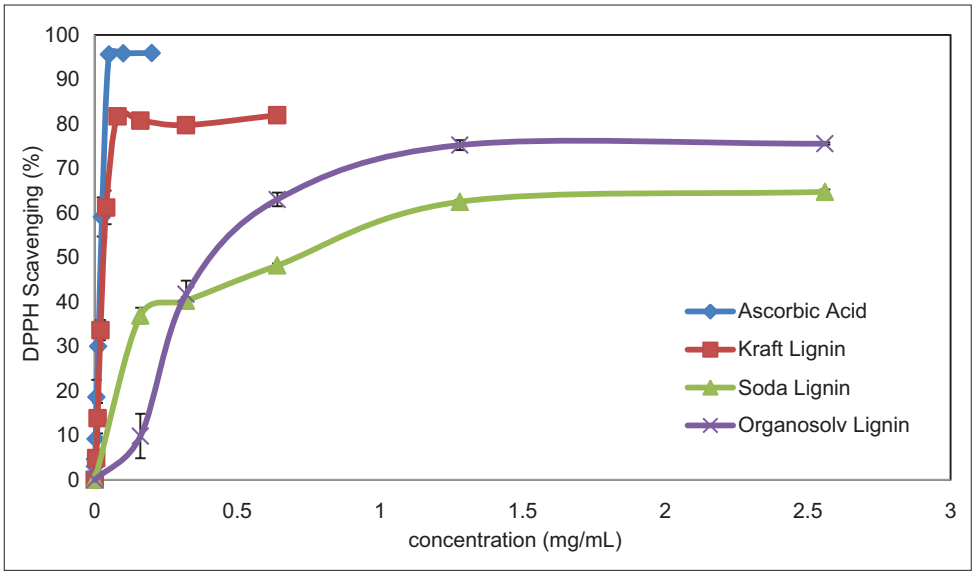

Fig.2: 2,2-diphenyl-1-picrylhydrazyl scavenging activities of oil palm empty fruit bunch lignin (soda, kraft, organosolv) compare with ascorbic acid. Results are expressed as a percent of mean ± standard deviation (n=3)

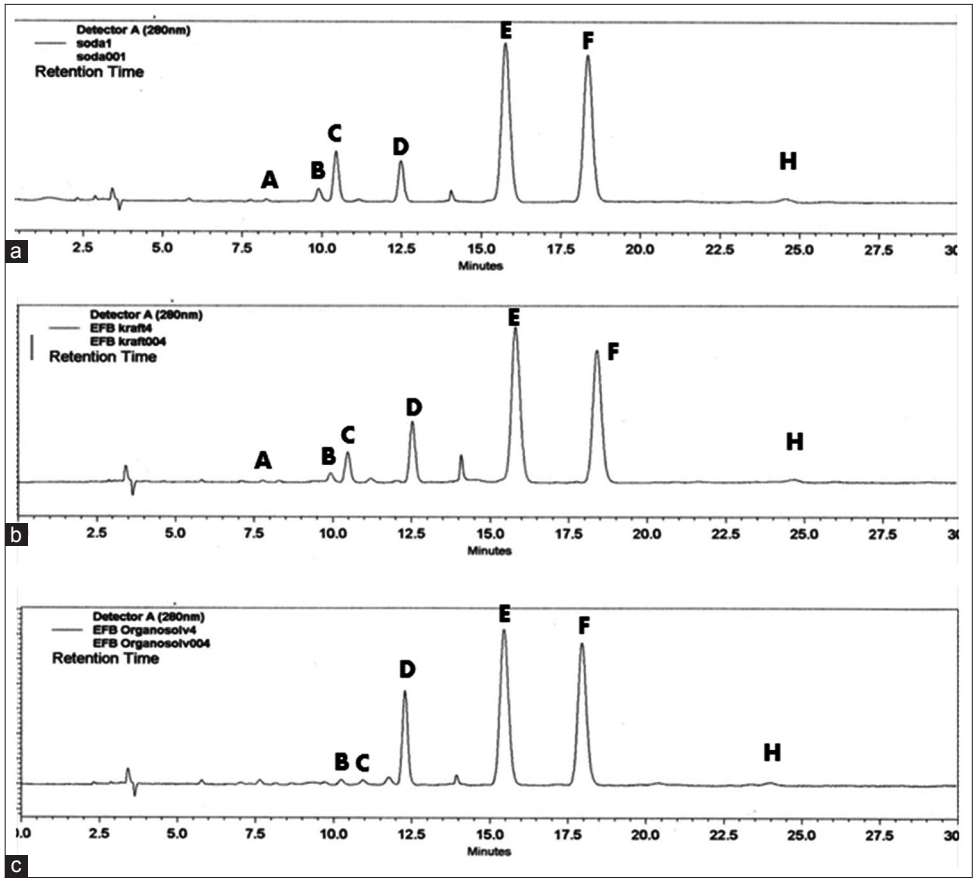

Fig. 3: Compounds (A: Hydroxybenzoic acid; B: Vanillic acid; C: Syringic acid; D: phydroxybenzaldehyde; E: Vanillin; F: p-coumaric acid; G: Syringaldehyde; H: Ferulic acid)that found in (a) soda (b) kraft and (c) organosolv oil palm empty fruit bunch lignin, (a): High-performance liquid chromatography chromatogram for soda oil palm empty fruit bunch lignin, (b): High-performance liquid chromatography chromatogram for kraft oil palm empty fruit bunch lignin, (c): High-performance liquid chromatography chromatogram for organosolv oil palm empty fruit bunch lignin

Table 3: The yield (% dry sample, w/w) of compounds from alkaline nitrobenzene oxidation of OPEFB lignin

Compound	% weight (dry basis)		
	Soda lignin	Kraft lignin	Organosolv lignin
Hydroxybenzoic acid (A)	0.064±0.002	0.034±0.015	ND
Vanillic acid (B)	0.294±0.003	0.159±0.009	0.025±0.006
Syringic acid (C)	0.885±0.009	0.426±0.008	0.054±0.003
p-hydroxybenzaldehyde (D)	0.283±0.004	0.353±0.006	0.551±0.022
Vanillin (E)	3.334±0.042	2.553±0.037	2.840±0.143
p-coumaric acid (F)	ND	ND	ND
Syringaldehyde (G)	7.074±0.126	5.109±0.007	5.803±0.281
Ferulic acid (H)	0.140±0.012	0.084±0.039	0.143±0.143
Molar ratio (G: S: H)	30:66:3	31:64:4	31:63:6
S/G ratio	2.20	2.06	2.10

*ND: Not detected, Results are expressed as mean±SD (n=3), OPEFB: Oil palm empty fruit bunch, SD: Standard deviation

their antioxidant effectiveness. The characterizations of all three types of OPEFB lignin were also conducted by ^{31}P NMR, GPC, HPLC, and flavonoids contents to investigate their correlation with GST activity and antioxidant effectiveness.

The quantitative^{31}P NMR were analyzed according to the method described by Granata and Argyropoulos [18]. The three lignin samples (soda, kraft, and organosolv OPEFB) were phosphitylated with TMDP in the presence of cyclohexanol as an internal standard. The concentration of each hydroxyl functional group (mmol/g) was calculated on the basis of the hydroxyl content of the internal standard and its integrated peak area (Table 1).

The overall amounts of phenolic OH in the region between 136 and 144 ppm (syringyl + guaiacyl + *p*-hydroxyphenyl) were increased in order of kraft > organosolv > soda OPEFB lignin. The high content of aliphatic hydroxyl group (AlkOH), in addition to the presence of several peaks between 145 and 150 ppm in the ^{31}P-NMR spectrum of all three types of OPEFB lignin were related to the presence of carbohydrate impurities which is suggested to be connected to the lignin fraction [25]. Pan *et al*. [10] also revealed that the AlkOH content has negative effect on antioxidant activity of the lignin.

Further, the weight average (M_w), number average (M_n), molecular weight, and polydispersity (M_w/M_n) of all three OPEFB lignin extracts were investigated using GPC. Acetylation was carried out in order to fully dissolve all OPEFB lignin samples in the chromatographic eluent (tetrahydrofuran). From Table 2, the lowest M_w and M_n values were obtained from kraft OPEFB lignin and this is due to the cooking process of the kraft OPEFB lignin. The hydroxide and hydrosulfide anions reacted with the lignin causing the polymer to fragment into smaller water/alkali-soluble fragment. The severe process of kraft pulping leads the lignin to break up into smaller molecular weights fragments [26]. Furthermore, all three OPEFB lignin have a relatively low polydispersity, which indicates that all lignins have a high fraction of low molecular weight [27]. The high molecular weight (M_n and M_w = number-average and weight-average molecular weights, respectively), enhancing heterogeneity, and polydispersity (M_w/M_n) are factors that decrease RSA [10].

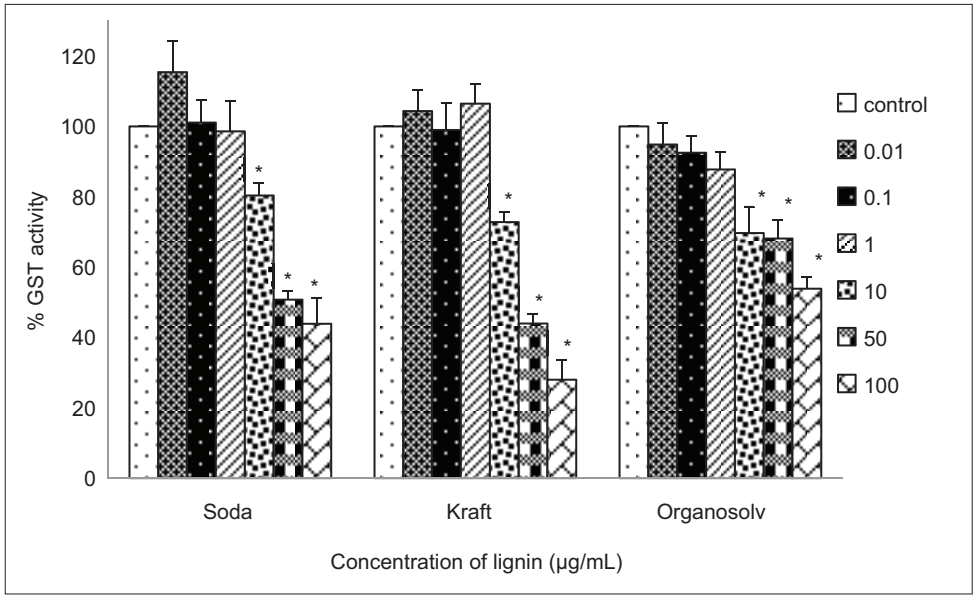

Fig. 4: *In vitro* effect of soda, kraft and organosolv palm empty fruit bunch lignin on glutathione-S-transferase enzymes activity. Results are expressed as percent of mean ± standard deviation (n=5), *Significantly different from control, p<0.05

Flavonoids including flavanols, flavones, and condensed tannins, are plant secondary metabolites. Consumption of the flavonoid-containing fruits and vegetables has been linked to protection against cancer and heart disease [28]. Although kraft OPEFB lignin showed a higher amount of total phenolic content than soda and organosolv OPEFB lignin, it showed less total flavonoid content than soda OPEFB lignin. This indicates that apart from flavonoids. Some other phenolic compounds were present in kraft OPEFB lignin.

Antioxidant activities of three OPEFB lignin and it main compounds were investigated by DPPH scavenging inhibition method. DPPH is a stable free radical. DPPH accepts an electron or hydrogen radical to become a stable diamagnetic molecule [29]. The DPPH percent RSA of the OPEFB lignin compared with ascorbic acid is shown in Fig. 2. Ascorbic acid is used as a positive standard because it is able to act as a good antioxidant.

Among the three types of OPEFB lignin, kraft OPEFB lignin with the lowest IC_{50}, exhibited the highest DPPH-RSA followed by organosolv OPEFB lignin. Meanwhile, soda OPEFB lignin with the highest IC_{50} value, exhibited the lowest DPPH-radical scavenging effect (Table 2). From this study, it is found that the antioxidant activity of all three OPEFB lignin was related to the phenolic hydroxyl group (ArOH) content and their M_w, $M_{n,}$ and M_w/M_n values.

Previous research by Barclay *et al*. [9], Dizhbite *et al.* [11], Ogata *et al.* [24] and Afandi *et al.* [30] had indicated that free phenolic hydroxyl group are essential for antioxidant activity. However, Acharya *et al*. [31] proved that, phenol and flavonoid contained in *Russula laurocerasi* showed strong correlation with antioxidant properties of the extract. From this study, it is proved that the antioxidant activity value is positively correlated to phenolic hydroxyl group (ArOH) content. In addition, the radical scavenging ability of phenolic compound depends on the ability to form a phenoxyl radical such as hydrogen atom abstraction and the stability of the phenoxyl radical [10]. The trapping and stabilization of radical by lignin was proposed by Barclay *et al*. [9]. Phenolic structures with substituents that can stabilize the phenoxyl radicals have higher antioxidant activity than those without.

Ortho substituents such as methoxy groups can stabilize phenoxyl radicals by resonance as well as hinder them from propagation. Conjugated double bonds can provide additional stabilization of the phenoxyl radicals through extended delocalization. However, a conjugated carbonyl group has a negative effect on antioxidant activity [10].

From Table 2, it is clear that the OPEFB lignin (kraft) with lowest molecular weight showed the highest antioxidant activity. This low molecular weight of kraft OPEFB lignin was produced from extensive depolymerization of lignin such as cleavage of ether linkages, which led to the formation of a new phenolic hydroxyl group (ArOH). Therefore, the low molecular weight kraft OPEFB lignin had the highest antioxidant activity compared to organosolv and soda OPEFB lignin.

The HPLC analyses show that syringaldehyde, vanillin, and *p*-hydroxybenzaldehyde were found to be the predominant compounds in soda, kraft, and organosolv OPEFB lignin. This had resulted from the degradation of non-condensed guaiacyl (G) and syringyl (S) units respectively [32]. All H, G, and S units were also present in each type of oil palm EFB lignin. The present result revealed that the OPEFB lignin is of the hydroxy phenol, guaiacyl, syringyl type which is the same as the oil palm frond lignin that had been reported by Hussin *et al.* [32].

From the HPLC analysis, vanillin and syringaldehyde were the main compounds in all three types of OPEFB lignin. Vanillin is widely used as an ingredient in food flavors, in pharmaceuticals and as a fragrance in perfumes and odor masking products [33]. Syringaldehyde has a similar chemical structure as vanillin and similar properties and because of this, it has a similar application. Pharmaceutical uses have been recently proposed, in which syringaldehyde is employed to synthesize drugs for cancer treatment [34,35].

Therefore, the presence of these valuable compounds in OPEFB lignin also promises an additional value to lignin especially in pharmaceutical and food supplement area. Before OPEFB lignin can be applied in the pharmaceuticals and food supplement development industries, the possibility of it interacting with other drugs or pharmaceutical agents was investigated.

The effect of OPEFB lignin on the inhibition of GST enzymes activity were in good correlation with the total flavonoid contents of each OPEFB lignin (Table 2). In addition, flavonoids have been shown to inhibit GSTs activity in human blood platelets, rat liver, and rat kidney [36]. Thus, this finding suggests that high GST inhibitory activity of OPEFB lignin

could be attributed to the high flavonoid content as indicated in the literature for the GST inhibitory effects of *Mitragyna speciosa* korth leaf extracts [37].

CONCLUSION

The antioxidant activity of soda, kraft, and organosolv OPEFB lignin extracts were evaluated in this study by determining their effects on DPPH RSA as well as their total phenolic, total flavonoids contents, number average molecular weight (M_n), and weight average molecular weight (M_w). Kraft OPEFB lignin was found to be a better antioxidant with IC_{50} values of 0.13 mg/mL followed by organosolv and soda OPEFB lignin with values of 0.45 mg/mL and 0.68 mg/g, respectively. The GST inhibitors have gained great importance because of their role in developing resistance to chemotherapy. OPEFB lignin extracts were therefore further investigated for their influence on GST enzymes activity in rat liver cytosolic fraction. All three types of OPEFB lignin were also investigated for their effects on GST enzyme activity. Among all OPEFB lignin extracts, soda OPEFB lignin was the most efficient inhibitor with IC_{50} value of 18.27±1.28 µg/mL followed by kraft OPEFB lignin with IC_{50} of 23.20±2.15 µg/mL. For organosolv oil palm EFB lignin extract, the IC_{50} value cannot be determined due to the percentage of inhibition being <50%. Findings suggest that the antioxidant activity of oil palm EFB lignin extracts have good correlation with total phenolic contents, M_n and M_w. On the other hand, the inhibition GST enzymes activity of OPEFB lignin has good correlation with total flavonoid content.

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