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ALKALINE-EXTRACTED LIGNIN FROM *XYLIA XYLOCARPA* AND ITS ANTIOXIDANT PROPERTIES

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Abstract: The composition of *Xylia xylocarpa* (Roxb.) Taub. sawdust showed its potential to be used as a lignin source. It is composed of almost 40% lignin, 20% glucan and 10% hemicellulose. In this work, lignin in the sawdust was extracted with potassium hydroxide (KOH) in three different conditions: (1) 0.5% KOH for 10 minutes, (2) 0.5% KOH for 30 minutes and (3) 1.5% KOH for 30 minutes. Then, the lignin was precipitated by a two-step precipitation. The highest lignin content was extracted and recovered with a high purity of 96.1% from the 0.5% KOH for 10 minutes condition, whereas the 1.5% KOH condition gave less lignin content. The ferric-reducing antioxidant power (FRAP) showed no significant values of all samples. The IC_{50} of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays showed that the lignin from the 1.5% KOH condition needed double compared to that of the other conditions to neutralize DPPH for 50%. The Fourier transform infrared spectroscopy results confirmed that the extracted lignin was mainly composed of guaiacyl and syringyl alcohol and the severity of the pretreatment affected the deformation of bonds in the lignin. Therefore, this short (10 minutes) and low KOH (0.5%) method could reduce the cost in biomass conversion.

Keywords: Lignin, *Xylia xylocarpa* sawdust, antioxidants, KOH pretreatment, sustainability.

Introduction

Lignin is one of the three major components in plant cell walls (Pan & Saddler, 2013). It is the second most plentiful natural compound after cellulose (Boudet & Grima-Pettenati, 1996). Lignin is a class of complex phenolic compounds of three monolignols, namely, *p*-coumaryl, guaiacyl (coniferyl) and syringyl (sinapyl) alcohol. Despite hydroxyl groups in its structure, lignin is primarily hydrophobic and acts as a cement for protecting and binding cellulose and hemicelluloses together in the plant cell wall (Donaldson *et al.*, 2017). Lignin can be soluble in hot alkali and condensable with phenol but it does not hydrolyze with acids (Patel & Parsania, 2018). Lignin from lignocellulosic biomass has shown to be a promising feedstock for chemical industries, as a precursor material for value-added compounds, e.g., benzene, toluene, xylene, phenol and phenolics (Isikgor & Becer, 2015; Zakzeski *et al.*, 2010).

Each phenolic acid is composed of one aromatic ring linked with hydroxyl and carboxyl groups. Due to its ability to donate hydrogen or a single electron, it can neutralize oxidative stress. This ability is called antiradical and/or antioxidant (Spiegel *et al.*, 2020; Vuolo *et al.*, 2019). The antioxidant property of a sample can be characterized using its ability to scavenge free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (a stable radical) (Brand-Williams *et al.*, 1995; Fadda *et al.*, 2014; Prevc *et al.*, 2013) and trolox equivalent antioxidant capacity ($ABTS^{+}$) method (cation radical) (Vuolo *et al.*, 2019) and/or the ability to inhibit the process of oxidation, such as the ferric-reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996; Spiegel *et al.*, 2020), copper reduction assay (chemical-based assay) (Apak *et al.*, 2005) and oxygen radical absorbance capacity assay (biological assay) (Prior & Cao, 1999).

Alkaline such as potassium hydroxide (KOH), sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$) and ammonium hydroxide (NH_4OH) has been widely applied in lignocellulosic-biomass pretreatment. The alkaline's ability of dissolution and saponification of ester bonds that link the components of lignocellulose cause the swelling and decrease in the polymerization and crystallinity of cellulose and the increase in the surface area of the treated biomass enhances the accessibility of substrates to enzymes (Bundhoo *et al.*, 2015; Y. Sun & Cheng, 2002). The hydroxide ion (OH^-) and metal ion (e.g., Na^+ and K^+) dissociate during pretreatment and the concentration of hydroxide ion directly increases proportional to the rate of the hydrolysis reaction (Kumar *et al.*, 2020). Although NaOH is significantly cheaper than KOH, KOH is a stronger base. Therefore, less amount of KOH is required for pretreatment. High amounts of sodium discharge might be environmentally harmful as it increases soil salinization and water pollution (Zheng *et al.*, 2014). By contrast, KOH discharge might benefit the environment as a fertilizer (Romero-Güiza *et al.*, 2017; Zheng *et al.*, 2014). Therefore, KOH could be a better option.

The wood processing industry and furniture production generate 155 kton/year of sawdust, equivalent to 10% of wood production or to energy 2 pJ/year (Yokoyama *et al.*, 2000). However, only a small portion of sawdust is used in the charcoal-making industry (Yokoyama *et al.*, 2000). *Xylia xylocarpa* (Roxb.) Taub var. kerrii (Craib & Hutch.) I.C. Nielsen is known as "Ironwood" (English name) and "Mai Daeng" (Thai name). It is one of the most popular hardwoods used in Thailand for construction. It is classified in the Leguminosae-Mimosoideae family. It is only one of the 12 species found in Southeast Asia, including India, Burma, Indo-China and Thailand. This deciduous and medium-sized tree can grow up to 25–40 m and has a bole straight and cylindrical branchless trunk for up to 12–25 m. The diameter of the lumber can reach 75–120 cm with a grayish to reddish flaky bark surface and pinkish color inside (Sosef *et al.*, 1998). The extract of *X. xylocarpa* which were obtained from using a series of organic liquids, shows antioxidant and antimicrobial properties (Lam *et al.*, 2016a; Nakmee *et al.*, 2016; Ramli *et al.*, 2018). Nakmee *et al.* (2016) extracted *X. xylocarpa* with sequential organic solvents, i.e., chloroform methanol, hexane, dichloromethane, ethyl acetate and methanol and then determined the antioxidant and antibacterial properties of the extracts. The antioxidant compounds are identified as a group of tannins, gallic acid, afzelechin and catechin polymer form (Nakmee *et al.*, 2016). The ethanolic extract of the stem, seed and bark of *X. xylocarpa* showed an antiradical scavenging activity of DPPH (Ramli *et al.*, 2018). The phenolic compounds in the methanolic extract of *X. xylocarpa* dried wood displayed anticholinesterases and memory-improving effects on amnestic-induced mice (Lam *et al.*, 2016b). To avoid using several expensive chemicals, in this paper, we show a quick lignin extraction from the sawdust of *X. xylocarpa* using KOH pretreatment. A two-step precipitation for lignin was applied and antioxidant properties, including FRAP and DPPH characterization and the Fourier transform infrared spectroscopy (FTIR) results were discussed.

Materials and Methods

X. xylocarpa Composition Analysis

Sawdust from *X. xylocarpa* was generously provided by furniture-making company Sompong in Nakhon Pathom, Thailand. The source of the tress was from Northern Thailand. The sawdust was milled with a hammer mill (Retsch, Germany) and then screened using a vibratory sieve shaker (Retsch Model AS 200, Germany) with 212 and 500 µm sieves. The sizes of 212 and 500 µm were used for composition analysis and pretreatment, respectively. The moisture content, ashes, water extract, ethanol extract, structural sugars and insoluble lignin content of the sawdust were determined following the laboratory analytical procedures by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2005a; Sluiter *et al.*, 2008; Sluiter *et al.*, 2005b). In brief,

for ash determination, 1 g of the sawdust was placed in a crucible and then burned at 575°C in a furnace (Chavachote Co., Ltd., Thailand) for 4 hours. Before weighting, the crucible was placed to cool down in a desiccator for 1 hour. The experiment was performed in triplicate.

For water and ethanol extraction, the sawdust was placed in a thimble before insertion into the Soxhlet tube. Two of 250 ml Erlenmeyer flasks with boiling stones were prepared before recording their weighs. 190 mL deionized (DI) water was added to the flask before connecting to the Soxhlet tube. The flask was heated using a hot plate. When a clear solution in the Soxhlet tubes was observed, the ethanol extraction was started. 190 mL of 190 proof ethanol was added in another prepared flask before being assembled to the Soxhlet tube. The heat was adjusted to provide a minimum of six siphon cycles per hour. When the reflux was completed, the extracted solid in the thimble was removed and transferred to a Buchner funnel with Whatman #1 filter paper before rinsing with 190 proof ethanol. The solid material was then air-dried. The solvent in the flasks was removed from the extract using a rotary evaporator equipped with a water bath and vacuum pump. The operation was conducted until the visible solvent disappeared. Then, the flasks were oven-dried at 70°C for 24 hours before cooling down in a desiccator and recording their weights.

Structural sugars and lignin content were determined by acid hydrolysis in the extracted solid. The acid-solubilized sugars were analyzed via high-performance liquid chromatography (Shimadzu Scientific Instruments, Columbia, MD) using Rezex™ RPM-Monosaccharide Pb^{+2} (8%) column and guard cartridges (Phenomenex Inc., Torrance, CA) with DI water as the mobile phase at 80°C and flow rate of 0.5 mL/min with the refractive index detector.

Alkali Pretreatments

The pretreatment was conducted. Ten g of total sawdust solid was added in 100 mL of 0.5% (w/v) of a KOH (ACS Reagent Chemicals, USA) solution in a 200 mL static Parr reactor. It was then heated to 150°C in a furnace (Chavachote, Thailand) for 10 minutes. This pretreatment was referred to as "0.5–10." Then, the concentrations of KOH and the pretreatment conditions were changed to 0.5% (w/v) of KOH for 30 minutes and 1.5% (w/v) of KOH for 30 minutes, which were then referred as "0.5–30" and "1.5–30," respectively. After the pretreatments, the reactor was immediately cooled down in ice-cold bath to stop the reaction. Each condition was done in triplicate before pooling all batches together for the separation process. The liquid and solid fractions were separated by a Buchner funnel with Whatman #1 filter and vacuum pump. The liquid part of each condition was collected for the precipitation process, whereas the solid part was discarded.

Lignin Recovery

The lignin in the liquid parts of the pretreatments was precipitated. The two-step precipitation method was modified from previous works (Pichainarong, 2002; R. Sun & Tomkinson, 2001). The pH of the pretreatment liquid was adjusted to equal 6 with glacial H_2SO_4 (Sigma-Aldrich, USA) and left to precipitate for 24 hours. The sediment was separated via centrifugation at 8000 rpm for 30 minutes. After centrifugation, the pH of the supernatant was adjusted to 3 with glacial H_2SO_4 and left to allow complete precipitation for another 24 hours. The pH 3 is optimum for insolubilizing lignin and settling down (Pichainarong, 2002). The precipitate was centrifuged at 8000 rpm for 30 minutes and collected. The collected lignin was dried in a hot-air oven at 80°C for 4 hours. The percentages of lignin extracted from the biomass were calculated using Equation (1):

$$\textit{Extractive lignin}\ (\%) = \frac{weight\ of\ extractive\ lignin}{total\ weight\ of\ lignin\ in\ biomass} \times 100 \quad (1)$$

Total Phenolic Compound Determination

250 mg of the precipitated lignin in 250 mL of 0.1 M boric acid (pH 12) was dissolved to make 1 g/L lignin solution. The total phenolic compound in the precipitated lignin

was determined using the Folin–Ciocalteu assay (Bamidele & Fasogbon, 2017). The Folin–Ciocalteu reagent (Sigma-Aldrich, USA) was composed of phosphomolybdic-phosphotungstic acid, which was reduced by the hydroxyl group in the phenolic compound as a result of the phosphotungstic-phosphomolybdic complex formation. The absorbance of the blue complex was measured at 630 nm using UV–visible spectrophotometer (Thermo Fisher Scientific, USA) and gallic acid was used as a standard. In brief, 30 µL lignin solution in 300 µL Folin–Ciocalteu reagent was added with 150 µL of 15% Na_2CO_3 (Sigma-Aldrich, USA) and 300 µL DI water. The reaction was kept in the dark for 30 minutes before measuring the absorbance.

Ferric-Reducing Antioxidant Power (*FRAP*) *Determination*

The FRAP assay was modified from a previous work (Benzie & Strain, 1996). The FRAP reagent was freshly prepared as follows: 300 mM sodium acetate buffer pH 3.6, 2.0 mM iron (III) chloride hexadehydrate ($FeCl_3$•$6H_2O$) (Sigma-Aldrich, USA) and 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, USA) in 40 mM HCl (ACS reagent, USA) in a ratio of 10:1:1. Before starting the assay, 3 mL of the newly made FRAP reagent was pre-incubated at 37°C for 5 minutes and then 400 µL of 0.25 g/L of lignin sample was added and mixed well. After 10 minutes, the absorbance of 595 nm of the reaction was determined using DI water as a blank and ferrous sulfate ($FeSO_4$•$7H_2O$) (Sigma-Aldrich, USA) as a standard. The measurements were conducted in triplicate for each sample.

Free Radical Scavenging Activity (DPPH) Determination

The free radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was modified from a previous work (Peschel *et al.*, 2006). In brief, freshly prepared 180 µL of 0.2 mM DPPH (Sigma-Aldrich, USA) in 99% ethanol was mixed with 20 µL of lignin solution in a 96-well plate and then kept in the dark for 30 minutes. The decolorization process was captured at 517 nm using ascorbic acid as a standard. The experiment was performed in triplicates for each sample. The percentage of inhibition (%inhibition) of DPPH was calculated using Equation (2):

$$\%inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

where $A_{control}$ is the absorbance of the control well (no lignin solution) and A_{sample} is the absorbance of the sample.

The results were further calculated as IC_{50}, which is the concentration of the sample that inhibits the free radical of DPPH to 50% from the starting concentration.

FTIR Determination

The FTIR spectra of the extractive lignin were determined with a PerkinElmer Spectrometer Frontier (Waltham, USA) with an attached ATR unit. The scanning wavenumber range was 4000–450 cm^{-1} with a resolution of 4 cm^{-1}. The grinded crystal lignin was pressed against the diamond ATR crystal top plate with the same pressure for all measurements. For each sample, 16 scans were obtained and averaged. Background scanning was performed regularly at 15 minutes intervals and the ATR correction was analyzed using the accompanying software package.

Results and Discussion

X. xylocarpa Compositions

The composition of *X. xylocarpa* sawdust was determined following NREL protocols. The results showed the potential of the sawdust as a lignin source. It was composed of ~40% (dw/w) lignin. Approximately 20% glucan and 10% hemicellulose were found (Table 1). Therefore, after dissolving the lignin of the sawdust, the solid part could be used as a substrate in enzymatic hydrolysis to generate sugars for the fermentation process. This method could increase the value of sawdust from *X. xylocarpa*

Table 1: Sawdust of the *X. xylocarpa* composition

Composition	Percentage (%)
Moisture content	5.59 ± 0.72
Ash	1.23 ± 0.13
Extractive compound	25.5
Glucan	19.02 ± 1.03
Xylan	7.28 ± 0.45
Galacturonan	0.00
Arabinan	0.94 ± 0.19
Mannan	0.00
Acid soluble lignin	10.08 ± 0.44
Acid insoluble lignin	28.83 ± 2.42
Total	**98.47** ± 2.71

± standard derivation of triplication

as a phenolic and carbohydrate resource for chemical and biochemical industries.

Lignin Recovery

The lignin compound in the sawdust was extracted with alkali in various conditions. Extracted lignin was precipitated from the pretreatment liquid by adjusting the pH. At pH 6, carbohydrates were insoluble (R. Sun & Tomkinson, 2001). After the first round of separation, the pH of the supernatant was adjusted to be 3 and left overnight to settle. Then, the precipitate was centrifuged, dried and collected. Surprisingly, the mildest condition, i.e., 0.5–10, could extract the highest lignin content (57.9%), whereas the most severe condition (1.5–30) gave the least lignin content (26.3%) (Table 2). The phenolic complex of lignin might be broken down as small molecules, which could not be settled and separated by the centrifugation condition. Lignin fractions with purities of 96.1%, 93.3% and 93.6% were extracted from samples put through conditions 0.5–10, 0.5–30 and 1.5–30, respectively. The purities of the lignin were similar to the lignin obtained from the black liquor of oil palm trunk fiber pulping (98.8%) (R. Sun & Tomkinson, 2001).

Total Polyphenolic Compound

The total polyphenolic compound was determined by the Folin–Ciocalteu assay. Although the Folin–Ciocalteu assay does not determine the total phenolic content, it assesses the reducing capacity of a sample to the reagent. The reagent reacts to all five subgroups of lignin: Phenolic acids, flavonols, flavanols, dihydrochalcones and flavanones (Platzer *et al.*, 2021). Therefore, this method is still a good option to quantify the total phenolic compound in a sample. The conditions

Table 2: Extracted lignin from the KOH pretreatment

Condition	Concentration of KOH (%)	Temperature (◦C)	Time (minutes)	Yield of Extractive Lignin (%w/w)	Purity of Lignin (%)
0.5–10	0.5	121	10	57.9	96.1
0.5–30	0.5	121	30	49.5	93.3
1.5–30	1.5	121	30	26.3	93.6

0.5–10 and 0.5–30 did not show significant differences in values, whereas the 1.5–30 condition sample had the lowest polyphenolic content (Figure 1). KOH is a strong base. Less concentration and briefer KOH pretreatment seem to provide enough strength to dissolve lignin from sawdust, which would be beneficial in reducing the operation cost. The high purity (93%–96%) of the lignin was obtained from applying the two-step precipitation method. However, small molecules of lignin for the most severe condition could not be precipitated.

Antioxidant Properties of the Extracted Lignin

The FRAP value showed the ability of the sample in reducing colorless Fe^{3+}-TPTZ complex into Fe^{2+}-TPTZ, which is a deep blue color (Spiegel *et al.*, 2020). The FRAP results showed no significant difference in the values of the three-pretreatment conditions. Furthermore, in the sample 1.5–30, the IC_{50} was 50% higher than those of the other samples. The samples 0.5–10 and 0.5–30 showed no significant difference in their values. The DPPH assay determines the ability of antiradicals to extract rich dihydrochalcones or flavanones (Platzer *et al.*, 2021). Therefore, the most severe condition might hydrolyze the structures of the lignin. The DPPH assay measures the ability of a sample to neutralize a stable radical ($DPPH^{\bullet}$) and to disappear as the result of decreasing of absorption (Brand-Williams *et al.*, 1995). IC_{50} values from the DPPH assay indicate the radicals in the sample reacting with reducers to become neutral molecules for 50%. Therefore, a low IC_{50} value is obtained from the higher antioxidant activity of the sample. However, the IC_{50} values from this research were 10 times higher than those of the lignin from the ethanol extraction of *X. xylocarpa*, which has been previously reported by Ramli *et al.* (2018). A direct comparison could not be performed because this work used five-time dilution of sample. The IC_{50} of the extract from organic solvents (dichloromethane, n-propanol and methanol) from softwood and hardwood were similar to the IC_{50} of this work (Ponomarenko *et al.*, 2014). However, the assay time of this work was longer. The heterogeneity of the lignin from the precipitation might also affect the IC_{50} value (Arshanitsa *et al.*, 2013).

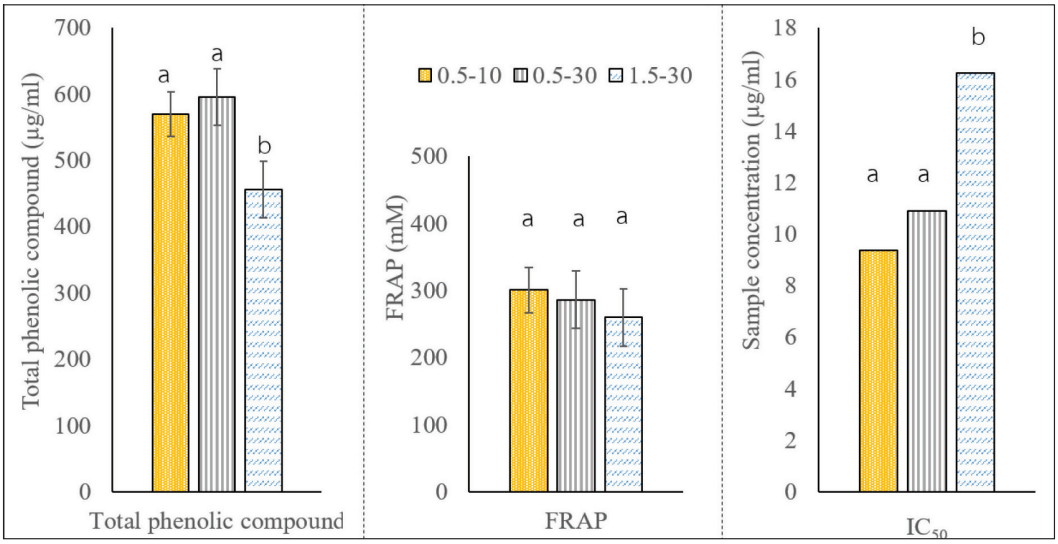

Figure 1: Total phenolic compound, FRAP and IC50 values of the samples from 0.5–10, 0.5–30 and 1.5–30 conditions. The different letters indicate statistically significant differences ($p < 0.05$). The error bars represent the standard deviations of triplication

FTIR Lignin Spectrum

In FTIR, a low transmittance at a wavelength means that there are more bonds that can be vibrated by energy corresponding to the incident light. Based on the results, the commercial lignin used as a control showed a different overall pattern from the samples. All of the lignin samples showed a similarity of peak patterns (Figure 2). The sample 0.5–10 had the highest number of chemical bonds, which could vibrate more energy, whereas the sample 1.5–30 showed the highest percent of the transmittance of the peaks. The spectrum at 3250 cm^{-1} represents O–H bonds from alcohols or phenols. C–H and C–H_2 asymmetric vibration (guaiacyl–syringyl) at 2930 cm^{-1} in commercial lignin was shaper than the peaks from the sawdust. The vibration of the bands assigned to the aromatic skeletal vibrations of lignin (C=C) at 1600 and 1508 cm^{-1} were obtained (Horikawa *et al.*, 2019). The peaks in the fingerprint region were assigned 1464 cm^{-1} for the C–H_2 deformation stretching in lignin and xylan and 1262 cm^{-1} for C–O stretch (lignin) and C–O linkage in guaiacyl aromatic methoxyl. The peak at 1230 cm^{-1} is often attributable to the syringyl ring and C–O stretching in lignin and xylan, that at 1040 cm^{-1} refers to the C–H in-plane deformation in the guaiacyl and C–O deformations in primary alcohol and that at 1106 cm^{-1} only showed in the sawdust–lignin samples, corresponding to cellulose and hemicellulos (C_2–O_2H) (Boeriu *et al.*, 2004; Horikawa *et al.*, 2019; R. Sun & Tomkinson, 2001).

The FTIR spectra confirmed that the extracts from the sawdust by alkali were mostly lignin (mainly guaiacyl and syringyl alcohol) and the severity of the pretreatment affected the deformation of bonds in the lignin. The sample 1.5–30, which was the most severe condition of the experiment, might lead to degradation of lignin as a result of the smaller number of complex bonds vibrated to the input energy.

Conclusion

The composition of the *X. xylocarpa* sawdust showed the potential to be used as a valuable lignin source. It was composed of almost 40% lignin, 20% glucan and 10% hemicellulose. The

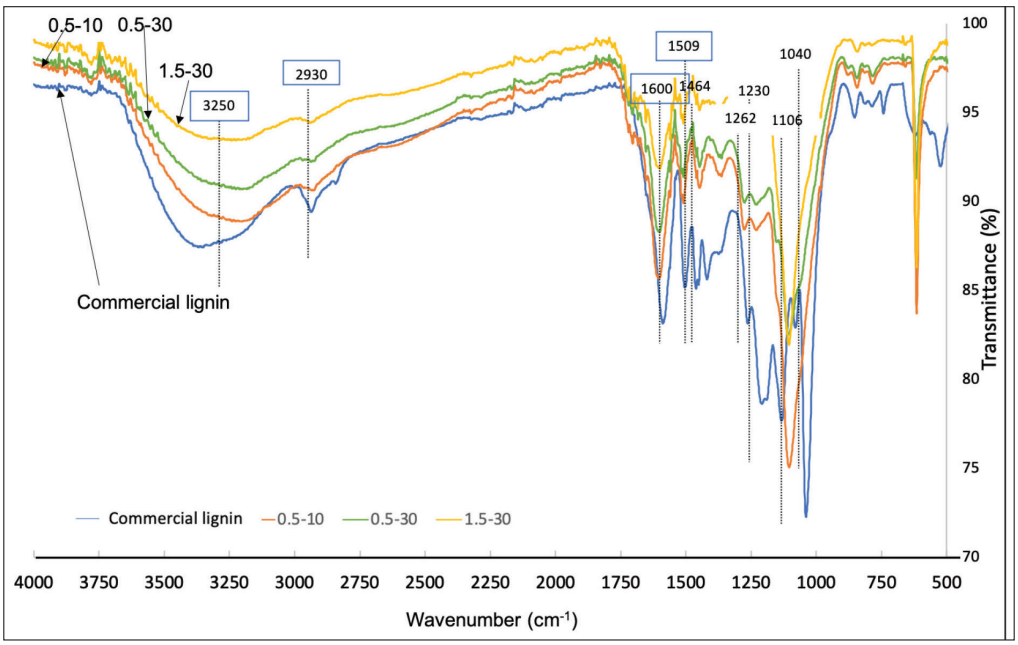

Figure 2: FTIR spectra of extractive lignin from *X. xylocarpa* with different conditions along with commercial lignin as the standard sample in the range of 450–4000 cm^{-1}

alkaline pretreatments were conducted with three severities: 0.5 M KOH for 10 minutes, 0.5 M KOH for 30 minutes and 1.5 M KOH for 30 minutes. Interestingly, the least severe condition gave the highest yield of lignin after a two-step acidic precipitation. The lignin showed antioxidant properties on the FRAP and IC_{50} of DPPH. The FRAP results showed no significant differences of values all samples, whereas the IC_{50} of DPPH of the most severe condition showed the least ability to neutralize DPPH. The FTIR results confirmed that the extracted lignin was mainly composed of guaiacyl and syringyl alcohol and the severity of the pretreatment affected the deformation of bonds in the lignin. Therefore, lignin from the sawdust could be obtained from the condition that only used 10 minutes with 0.5 M KOH pretreatment. This short time (10 minutes) and low concentration of KOH (0.5%) method would be a highly beneficial in reducing the pretreatment operation cost in biomass conversion.

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