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Antioxidant and antimicrobial activities of lignin-derived products from all steam-exploded palm oil mill lignocellulosic biomass waste

Sholahuddin Sholahuddin[1,2*], Dian Yosi Arinawati[3,5], Vinod Kumar Nathan[4,5], Chikako Asada[5] and Yoshitoshi Nakamura[5*]

Abstract

Background Steam explosion pretreatment has been proven to be an effective treatment for breaking down the recalcitrant character of lignin–carbohydrate complexes (LCC) in lignocellulosic biomass. This study investigated the production of lignin-derived products from steam-exploded palm oil mill lignocellulosic biomass waste (POMLBW), that is, empty fruit bunches (EFB), kernel shells (KS), and kernel fibers (KF), also known as mesocarp fibers. Steam explosions cause lignin depolymerization, which forms various polyphenols. The low average molecular weight of the steam-exploded lignin-derived products and their antioxidant activities could potentially enhance their antimicrobial activities.

Methods POMLBW was steam-exploded with various degrees of severity factors (R_0: 4.03, 4.91, 5.12, 5.35, and 5.65). Steam-exploded POMLBW produces lignin-derived products such as low-molecular-weight lignin (LML) and water-soluble lignin (WSL). Antioxidant activity was evaluated using 0.5 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Polyphenol content was evaluated using the Folin–Ciocalteu method. The antimicrobial activity was evaluated using an agar diffusion assay with Gram-positive and Gram-negative bacteria, and the thermal characteristics were evaluated using differential scanning calorimetry and thermogravimetric analysis.

Results WSL and LML resulted in high radical scavenging activity (RSA) of approximately 95% and 80%, with 0.25 g/L and 0.5 g/L of EC50, where the polyphenol amount was 242–448 mg/g (catechin eq.) and 20–117 mg/g (catechin eq.) under all LML and WSL conditions, respectively. The steam-exploded POMLBW had an average molecular weight of 1589–2832 Da, and this condition, including high RSA and polyphenol amounts, was responsible for the high antimicrobial activities of LML against both Gram-positive (*Salmonella enterica, Pseudomonas aeruginosa, and Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria. Additionally, the thermal properties investigations revealed that the glass transition temperature was 80–90 °C (*T*g), the melting temperature (T_m) was 338–362 °C, and the start temperature was 101–128 °C at the beginning of mass loss.

Conclusions These results show that the lignin-derived product from steam-exploded POMLBW has the potential for antioxidant (LML and WSL) and antimicrobial (LML) applications with good thermal resistance.

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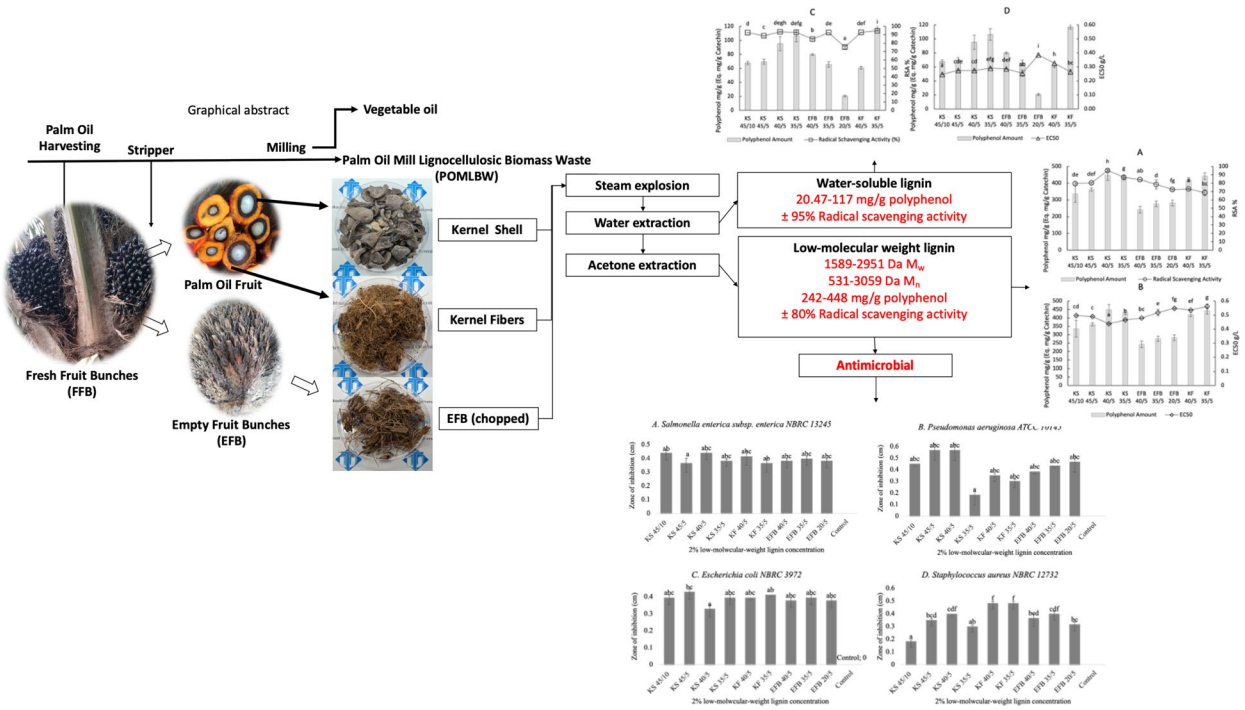
Keywords Steam explosion, Low-molecular-weight lignin, Antioxidants, Antimicrobials, Palm oil mill biomass waste, Biomass conversion, EFB, Kernel shell, Kernel fibers

Graphical Abstract



Introduction

The palm oil industry produces large amounts of lignocellulosic biomass. Palm oil mill lignocellulosic biomass waste, such as empty fruit bunches, kernel shells, and kernel fibers, also known as mesocarp fibers, can be valorized not only for energy conversion but also for sustainable materials. Abundant lignocellulosic biomass waste can potentially be used as a sustainable material, such as in lignin-derived products [1]. Lignin-derived products are widely reported to have several abilities, such as UV-blocking agents [2–4], flame retardants [5, 6], antimicrobials, and antioxidants [7–11]. Steam explosion pretreatment has been reported to break down the lignin–carbohydrate complex (LCC) polymer chain in lignocellulosic biomass. As a physicochemical pretreatment, steam explosion not only changes the physical, but also the chemical structure of the biomass [12]. The steam explosion pretreatment breaks the lignin polymer into nanosized products and produces lignin-derived products such as lignin precursors, including sinapyl alcohol, p-coumaric alcohol, and coniferyl alcohol. Furthermore, steam explosion pretreatment can form phenolic compounds with increasing severity factors. In addition, depolymerization continuously breaks down the lignin precursors into catechol, guaiacol, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and vanillic acid [13]. Lignin-derived products are produced in several biorefinery processes and are characterized by their antimicrobial and antioxidant activities. Phenolic compounds produced from steam-exploded biomass have been reported to have good antioxidant activity with satisfactory radical scavenging activity [14]. These products are contained in low-molecular-weight lignins (LML) and water-soluble lignins (WSL) as lignin-derived products from steam explosion pretreatment [13].

The antimicrobial mechanism of lignin-derived products involves lysis caused by polyphenols, which can damage the cell wall by leaking internal fluids. Furthermore, reactive oxygen species (ROS) absorbed by lignin-derived polyphenols with high antioxidant activities can alter normal redox physiology by releasing and inducing

oxidative stress upon contact with bacteria [15]. Alternatively, nanosized lignin-derived polyphenols can penetrate bacterial cells via the Trojan horse mechanism. In this process, the monophenolic compound from lignin can deplete adenosine triphosphate (ATP), which infiltrates bacteria and decreases the pH (intracellular). Several reports have stated that lignin-derived phenolic compounds can inhibit microbial growth, mostly because of their phenolic functional groups and side-chain structure. The lignin-derived phenolic compound contains a methyl group and a double bond that has a stronger biocide effect compared to the oxygen-containing functional groups, such as the carbonyl, ester, and hydroxyl groups, which are responsible for its antioxidant behavior [4]. Moreover, the small size and high purity of low-molecular-weight lignin enhance its antimicrobial activity because of its ability to penetrate microbial cells [16]. This study aims to evaluate the production of lignin-derived products from agricultural biomass waste via steam explosion pretreatment of all lignocellulosic biomass waste of palm oil mills. The steam-exploded lignin-derived products were characterized for their thermal behavior and antioxidant and antimicrobial activities to evaluate the potential of the valorization process of biomass waste in the agriculture industry.

Materials and methods

Material

The palm oil mill lignocellulosic biomass waste used in this study consisted of empty fruit bunches, kernel fibers, and kernel shells collected from a small palm oil mill in Riau Province, Sumatra Island, Indonesia. The EFB was chopped to 3–5 cm in length, KS was approximately 1–3 cm in diameter, and KF was approximately 5–10 cm. All the samples were air-dried at 65 °C for 24 h to avoid fungus growth.

Steam explosion pretreatment

Steam explosion pretreatment conditions were set under various conditions (pressure and residence time) according to the natural hardness of the biomass. EFB was subjected to 20 atm (213 °C) for 5 min of residence time (20/5), 35 atm (243 °C) for 5 min (35/5), and 40 atm (250 °C) for 5 min (40/5). Because KS was the hardest biomass waste from the POMLBW, it was subjected to relatively higher pressures and residence times of 35 atm for 5 min (35/5), 40 atm for 5 min (40/5), and 45 atm (258 °C) for 5 and 10 min (45/5 and 45/10). KF was subjected to 35 atm for 5 min (35/5) and 40 atm for 5 min (40/5). The steam explosion was conducted using a 2-L reactor (NK-2L, Japan Chemical Engineering and Machinery Co. Ltd., Osaka, Japan) regulated by deionized water. The severity factor (R0) from the steam explosion pretreatment was calculated using the following formula:

$$R_0 = t.\exp^{[(T_r-100)/14.75]}.$$

T_r is the temperature reaction (°C), and t is the resident time [17]. The severity factors for 20/5, 35/5, 40/5, 45/5, and 45/10 were 4.03, 4.91, 5.12, 5.35, and 5.65, respectively. For comparison, the initial conditions for all POMLBW were milling treatment using a crusher mill (Wonder Crush Mill D3V-10, Osaka Chemical Co., Ltd., Japan) for one minute for all samples.

Extraction

The general process for the production of lignin-derived products from steam-exploded POMLBW and the characterization process are shown in Fig. 1. The steam-exploded POMLBW was extracted using 60 mL/g distilled water for 24 h at room temperature; the water-soluble-derived lignin (WSL) was then filtered using filter paper no. 131 (diameter 185 mm; Advantech Co., Ltd., Tokyo, Japan). To prepare the sample for antioxidant activity and polyphenol measurement, the water-soluble material was frozen at − 80 °C for 24 h, vacuum freeze-dried (Eyela FDU-1200) at − 50 °C and stored at − 30 °C.

The water-insoluble material was subjected to acetone extraction to extract LML. Acetone extraction was stirred at 300 rpm for 3 h at room temperature and filtered. The

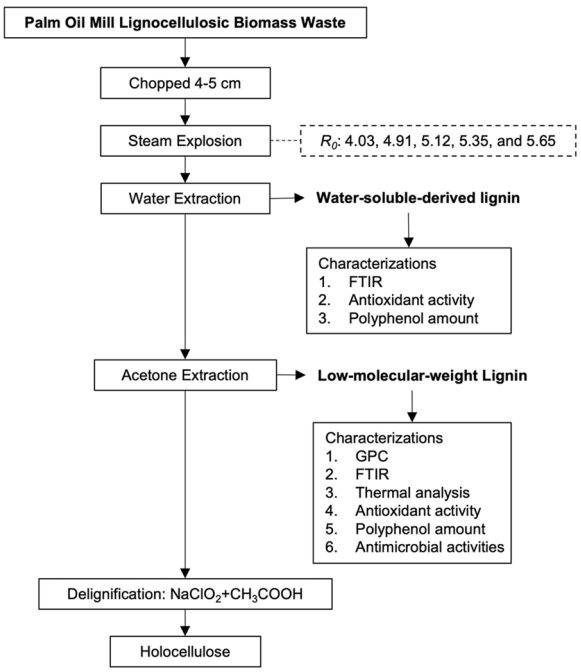


Fig. 1 Lignin-derived products' biorefinery and characterization flow-chart

subsequent acetone-soluble material was further subjected to an evaporation process using an evaporator (Eyela N-100, Shanghai, China) and low-temperature circulator (Eyela CA-1113, Shanghai, China) and submerged in a 60 °C water bath (EW-100RD, As-One, Osaka, Japan). To obtain the LML, the acetone-soluble material was vacuum oven dried (Vacuum oven ADP300, Yamato, Tokyo, Japan) at 45 °C for 24 h, and the vegetable oil from POMLBW was separated using filter paper as an oil trap under the aluminum cup by dripping process (30° slope). The LML remained in the evaporator glassware, while the oil dripped into the trap cup and weighed as vegetable oil.

The acetone-insoluble material was then mixed with 60 mL/g deionized water to evaluate the holocellulose (cellulose and hemicellulose), and the delignification process was continued using 0.25 g/l sodium chlorite (NaClO2) and 0.05 mL/g acetic acid (CH3COOH) at 80 °C. Sodium chlorite and acetic acid were added every hour, and this was repeated four times. The holocellulose was then separated using vacuum filtration (WJ-20, Shibata, Saitama, Japan) with a fiberglass filter GF-75, 110 mm (Advantech Co., Ltd., Tokyo, Japan) and transferred to the oven at 105 °C for 24 h to obtain the amount of hemicellulose. The sodium chlorite-soluble material was evaluated by oven drying at 105 °C until no more liquid remained and weighed as a lignin representative (Klason lignin) after deducting the amount of sodium chlorite and acetic acid that had been used.

Gel permeation chromatography (GPC) for molecular weight analysis

The molecular weight of LML was characterized via GPC (Shimadzu detector UV-VIS SPD-20A, pump LC-20AD, deaerator DGU-12A, and oven CTO-20AC) using 5 mg of LML mixed with 5 mL of tetrahydrofuran and operated using 10 µl of samples into column sets Shodex GPC LF-G, Shodex GPC LF-804, Shodex GPC KF-800D, and Shodex GPC KF-801 with tetrahydrofuran mobile phase, with a flow of 0.6 mL/min, 40 °C.

Fourier transform infrared (FT-IR) spectroscopy

Fingerprinting was performed using FT-IR spectrum two (PerkinElmer, MA, USA) at 400–4000 cm^{-1} with 32 scans for WSL and LML using 5 mg samples.

Polyphenol amount and antioxidant activities

The polyphenol content and antioxidant activity of both WSL and LML were evaluated. The antioxidant activity was evaluated by measuring hydrogen/electron donation ability using 0.5 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, based on a change in absorbance, indicating a decrease in DPPH. Freeze-dried water-soluble material (100 mg) was prepared for antioxidant activity analysis, dissolved in 99.5% ethanol (100 mL), and homogenized using an ultrasonic processor for 2 min (Hielscher, UP100H, Germany). Furthermore, the scavenging activity was evaluated using 1 mL samples with 1, 2, 5, 10, and 20 dilutions. The different dilutions were mixed with 1 mL of ethanol and 1 mL of 0.5 mM DPPH in an ethanol solution, placed in a brown test tube, and submerged in a 30 °C water bath for 30 min. The change in absorbance was evaluated using a microplate reader (Tecan, Infinite 200 PRO, read with I-control) using a 200 µl sample in a 96-well plate. The wavelength was set to 517 nm and calculated using the following equation:

$$\text{Radical schavengin activity}(\%) = \left[\frac{A_0 - (A - A_a)}{A_0}\right].$$

The absorbance of the sample and DPPH after a 30-min reaction was *A*. The absorbance of DPPH was *A0*, whereas that of the untreated sample was *Aa*. All samples were examined in triplicates.

Polyphenol content was evaluated using the Folin–Ciocalteu method. LML and freeze-dried WSL (100 mg) were mixed with 100 mL distilled water and homogenized using an ultrasonic processor for 2 min (Hielscher, UP100H, Germany). The mixture was divided into 200 µl samples and diluted into 1, 2, 5, and 10, added with 1 mL of Folin–Ciocalteu, 4 mL of distilled water, and 1 mL of 10% W/V sodium carbonate, mixed for 10 s, and reacted for one hour. The amount of polyphenol was measured by a microplate reader (Tecan, Infinite 200 PRO, read with I-control) using a 200 µl sample in a 96-well plate. The wavelength was set at 760 nm, and the results were expressed as the amount of catechin per mg of the sample.

Antimicrobial activities of LML

The antimicrobial activity of LML was evaluated using an agar diffusion assay with Gram-positive (*Staphylococcus aureus* NBRC 12732) and Gram-negative (*Pseudomonas aeruginosa* ATCC 10145, *Salmonella enterica subsp. enterica* NBRC 13245, and *Escherichia coli* NBRC 3972) bacteria. The bacteria were cultured using nutrient broth (8 g/L) and incubated for 24 h at 37 °C. The agar diffusion assay was performed on Mueller Hinton Agar (MHA). 100 µl of 2% LML was diluted with N,N-dimethylformamide (DMF) was placed in vertically punched holes on the media, incubated at 37 °C for 24 h, and evaluated for the inhibition zone. DMF was used as the control.

Thermal analysis of LML

To measure the glass transition temperature (T_g), thermal analysis was conducted on 5–5.5 mg dried LML using

differential scanning calorimetry (DSC; Exstar 6000, SII Seiko, Tokyo, Japan) set to 30–550 °C with 20 °C/min temperature flow. Nitrogen was injected nitrogen at 50 mL/min. The weight loss ratio (%) from thermogravimetry (TG) of LML was evaluated using a thermogravimetric analyzer (TG/DTA SII EXSTAR6300; Seiko Instruments Inc.) from 20 to 800 °C with a 5 °C/min heating rate, and injected with 200 mL/min nitrogen gas. The limiting oxygen index (LOI) of the samples was determined according to the ASTM D2863 [18].

Statistical analysis

Single-factor ANOVA was used to compare the results, and 5% Duncan's multiple range test (DMRT) was used as the post hoc test using Microsoft Excel.

Results and discussion

Steam-exploded POMLBW mass balance

Component analysis (Fig. 2) revealed a general compound in the milling-treated and steam-exploded POMLBW. The holocellulose (cellulose and hemicellulose) contents from steam-exploded EFB were 70.22, 42.91, and 37,71% for pressure and steaming times of 20/5, 35/5, and 40/5, respectively, with 64,11% from the milling treatment alone. The WSL from the steam-exploded EFBs increased by 183.58% (EFB 35/5) from the milling treatment. Furthermore, the NaClO2-soluble material, Klason lignin, was 8.05, 12.82, 28.96, and 30.52% for the 20/5, 35/5, 40/5, and milling treatments, respectively. The remaining vegetable oil was only contained in the EFB, which yielded 6.93, 10.18, 11.28, and 6.71% from the 20/5, 35/5, 40/5, and milling treatments, respectively. The holocellulose content of KF was 29.38, 26.6, and 68.27% for the 35/5, 40/5, and milling treatments, respectively. The WSL increased by 213.24% (35/5) compared to that of the milling treatment. The NaClO2-soluble material from KF was 27.16, 42.68, and 40.8% after the 35/5, 40/5, and milling treatments, respectively. Moreover, the holocellulose contents of KS were 37.41, 32.54, 35.73, 18.84, and 36.44% in the 35/5, 40/5, 45/5, 45/10, and milling treatments, respectively. The steam explosion increased the WSL content fivefold compared to the milling treatment alone, with no significant difference between the pressure and steaming time. The $NaClO_2$-soluble materials of KF were 21.2, 20.83, 35.63, 20.07, and 43.47% for the 35/5, 40/5, 45/5, 45/10, and milling treatments, respectively. The morphology of EFB 20/5 maintained its macrofibril form with 70.22% holocellulose content. The LML production increased with the severity factor, indicating that the steam explosion effectively separated the lignin from the LCC into LML and WSL. Because of the small amounts of WSL and LML in all milling-treated samples, the milling treatment was discontinued for all characterization processes.

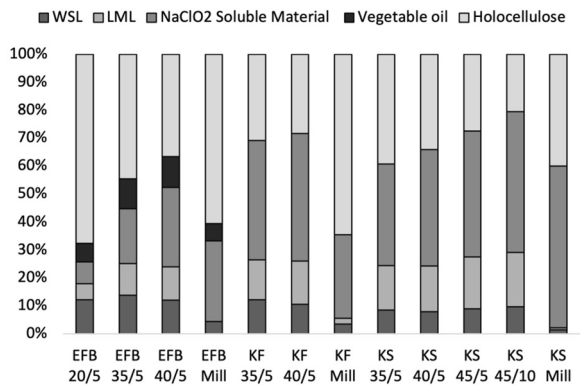


Fig. 2 Mass balance of milled and steam-exploded POMLBW

LML molecular weight

Steam-exploded POMLBW produces an acetone-soluble low-molecular-weight lignin. Steam-exploded EFB 20/5, 35/5, and 40/5 steam-exploded EFBs produced 6.04, 10.92, and 12.35% LML, respectively. KF 35/5 and 40/5 produced 13.58 and 14.59% LML, respectively, while KS 35/5, 40/5, 45/5, and 45/10 produced 15.11, 15.74, 17.45, and 17.48 LML, respectively (Fig. 2). GPC analysis of the LML showed that the average molecular weights (M_w) of the steam-exploded 20/5, 35/5, and 40/5 EFB pretreatments were 1589, 2470, and 2951 Da, respectively, and the average molecular numbers (M_n) were 531, 764, and 799, respectively. The average M_w of the LML from steam-exploded KS was 2445–2832 Da, and the average M_n was 714–766. The steam-exploded KF pretreatments produced LML with an average M_w between 2769 and 1840 Da and M_n 757 and 621 Da for 35/5 and 40/5, respectively (Table 1). Sun et al. reported that the samples were precipitated under low-pH conditions (pH 2) using hydrochloric acid (HCl) and extracted using dichloromethane (CH_2Cl_2) to obtain a molecular weight of 2790 from black liquor (AQ pulping) EFB [19]. Other reports suggest that complex catalytic depolymerization using a variety of catalysts, such as Al_2O_3, SiO_2, SiAl, MgO, H, Hb, and HZSM-5, is required to extract LML with an average molecular weight ranging from 1431 to 2797 Da [20]. In addition, steam explosion acid catalysis has resulted in M_w of 2147–2357 Da with 5909–10947 Da of M_n [20]. In addition, the steam-explosion acid-catalyzed reaction resulted in M_w of 2147–2357 Da with 5909–10947 Da of M_n [21]. In contrast, our results demonstrate that the water-only steam explosion pretreatment is an effective and simple process for producing LML from POMLBW using only water and acetone extraction, which includes an environmentally friendly pretreatment

Table 1 Gel permeation chromatography (GPC) of acetone-soluble material as low-molecular-weight lignin

	Empty fruit bunches			Kernel shell				Kernel fibers	
	20/5	35/5	40/5	3/55	40/5	45/5	45/10	35/5	40/5
Severity factor (R_0)	4.03	4.91	5.12	4.91	5.12	5.35	5.65	4.91	5.12
Average Weight of Molecular Weight (M_w)	1589	2470	2951	2445	2832	2655	2675	2769	1840
Average number of molecular weight (M_n)	531	764	799	714	766	721	728	757	621
M_w/M_n	2.99	3.23	3.69	3.42	3.70	3.68	3.67	3.66	2.97
Average size of molecular weight (M_z)	3687	5750	6883	5390	6476	6135	6070	6576	4134

for biomass. This non-complex refinery, using steam explosion as a pretreatment for POMLBW, continues the lignin-derived product separation process using only water and acetone at room temperature without complex processes and harmful chemicals, which could result in a high percentage of LML and WSL. Moreover, the LML has a small molecular size (1.5–2.8 nm by conversion), which could support the requirement for antimicrobial activity.

LML and WSL FT-IR fingerprinting

FT-IR revealed the fingerprinting of WSL (Fig. 3). FT-IR evaluation suggested the presence of a functional group with an IR band at 3222–3261 cm^{-1}, which could be attributed to O–H stretching and H-bonds for alcohols and phenols [22], appeared in all steam-exploded POMLBW. The IR band 2935 cm^{-1}, which is attributed to C-H stretching (asym.) Alkanes and O–H stretch carboxylic acid appeared in all the steam-exploded EFB and 40/5 KF pretreatments. The IR band at 1583 cm^{-1}, attributed to C=C double-band stretching, appeared in all steam-exploded POMLBW, and 1553 cm^{-1}, assigned to C=N [22], appeared in the 35/5 pretreatment of KS. The IR band at 1388 cm^{-1}, assigned to the isopropyl (–C(CH_3)2) group due to CH_3 and CH_2 bending, also appeared in all the steam-exploded POMLBW. The IR band at 1275 cm^{-1} was linked to the asymmetric stretching vibrations of the C–O–C linkages in phenolic ethers (C–O stretch) and phenolic hydroxyls or esters that appeared in the EFB 35/5 and 40/5, KS 45/5 and 45/10, and KF 40/5 pretreatments. The IR band at 1244 cm^{-1}, assigned to the C-O asymmetric stretching of COOH, appeared in the EFB 20/5, KS 35/5 and 40/5, and KF 40/5 pretreatments. The IR band at 1159 cm^{-1} was assigned to Aromatic C-H

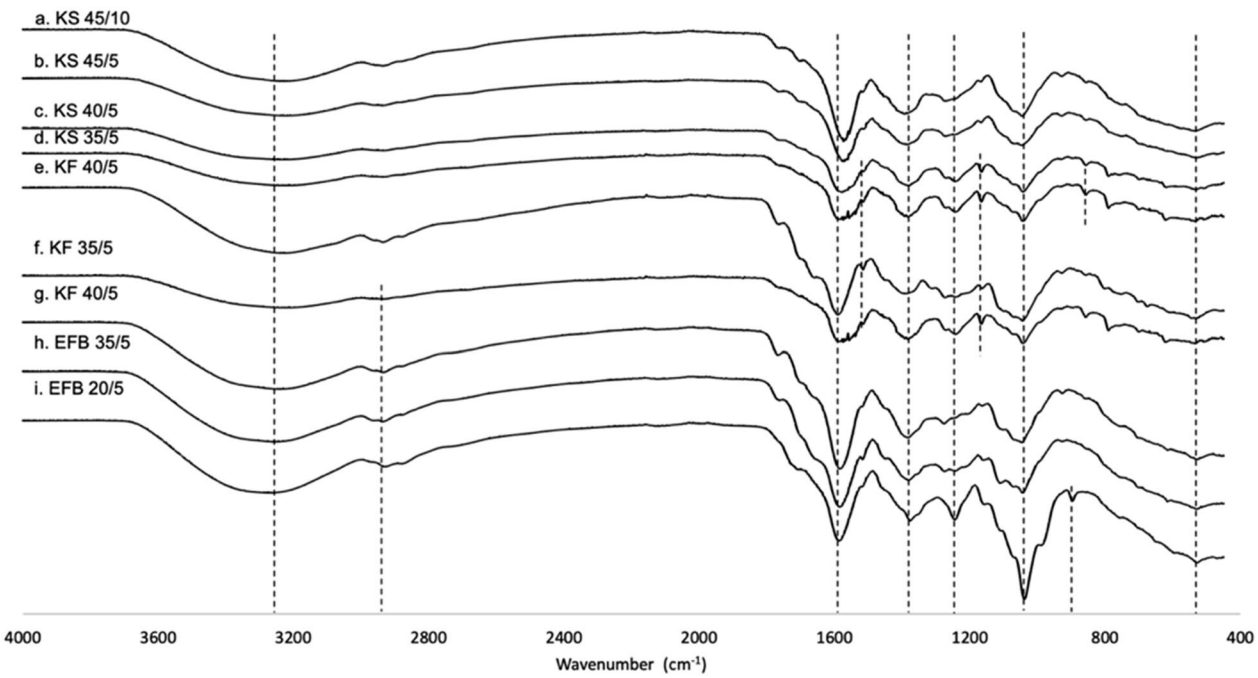


Fig. 3 FT-IR WSL from different steam-exploded (SE) pretreatments (steam pressure/steam time) of kernel shell (KS), kernel fiber (KF), and empty fruit bunches (EFB). **a** KS 45/10, **b** KS 45/5, **c** KS 40/5, **d** KS 35/5, **e** KF 40/5, **f** KF 35/5, **g** EFB 20/5, **h** EFB 35/5, **i** EFB 40/5

in-plane deformation from Guaiacyl Syringyl L [23] that appeared in KS 35/5 and 40/5, and KF 35/5 and 40/5, respectively. The 1045 cm^{-1} band, assigned to methoxyl groups appeared in all steam-exploded POMLBW, also appeared at 1035 cm^{-1} for aromatic C–H in-plane deformation from Guaiacyl L [23].

Figure 4 shows the FT-IR spectra of all LML from POMLBW. The IR band at 2938 and 2848–2849 cm^{-1} suggest the functional groups C–H asymmetric ($–CH_3$) and symmetrical stretching vibration from methyl groups ($–CH_3$), methine groups (–CH–), and a methylene group ($–CH_2$) with strong intensity in the all-steam-exploded POMLB. The aliphatic methylene group C–H (IR band at 2916 cm^{-1}) showed strong intensity in the EFB 35/5, 40/5, and KS 35/5 pretreatments. Carbonyl stretching of unconjugated ketones and a carbonyl group IR band at 1711 and 1737 cm^{-1} from Guaiacyl L and Guaiacyl Syringyl L [23], respectively, appeared with strong intensity under all treatment conditions (EFB, KS, and KF). O–CH3 deformation, CH_2 scissoring, and guaiacyl/syringyl ring vibrations at 1452 and 1455 cm^{-1} [23] appeared with strong intensity under all conditions of EFB, KS, and KF. Syringyl ring breathing with CO stretching and C–O stretching for secondary alcohols from 1213 and 1120 cm^{-1} from the guaiacyl (G) unit appeared in all steam-exploded POMLB with strong intensity, except for KS 35/5, which showed medium intensity. The aromatic C–H in-plane deformation IR band at 1031–1033 cm^{-1} [24] appeared in KS 35/5 and 45/5 with strong intensity. Meanwhile, the aromatic C–H out-of-plane in positions 2 and 6 of the syringyl (S) and p-hydroxyphenyl (H) units from 720 to 940 cm^{-1} appeared for EFB 35/5 under all steam-exploded KF and KS conditions with strong intensity. This fingerprinting revealed hydroxyphenyl, syringyl, and guaiacyl groups, which are monolignols, as the lignin-derived products. These monolignols are substructured by phenolics such as p-hydroxyphenyl from coumaryl alcohol, syringyl from sinapyl alcohol, and guaiacyl from alcohol moieties [25, 26]. Phenolic compounds (functional groups) fragmented with oxygen, such as –OH, CO, and –COOH, are considered to have antimicrobial capabilities [16] (Figure 5).

LML and WSL polyphenol amount and antioxidant activity

The polyphenol precursors of the 2-pyrone-4,6-dicarboxylic acid (PDC), such as p-hydroxybenzoic acid, gallic acid, p-hydroxybenzaldehyde, syringic acid, vanillic acid, vanillin, p-coumaric acid, protocatechuic aldehyde, and ferulic acid, have been reported from EFB, KF, and KS, which included low-molecular-weight phenolic compounds by using subcritical water extraction from 100 to 200 °C [27]. These lignin-derived products can be fractionated by steam explosion pretreatment [28], and the resulting lignin precursors can be examined for their antioxidant activity. The antioxidant activity of lignin-derived products, such as LML, was widely reported using DPPH

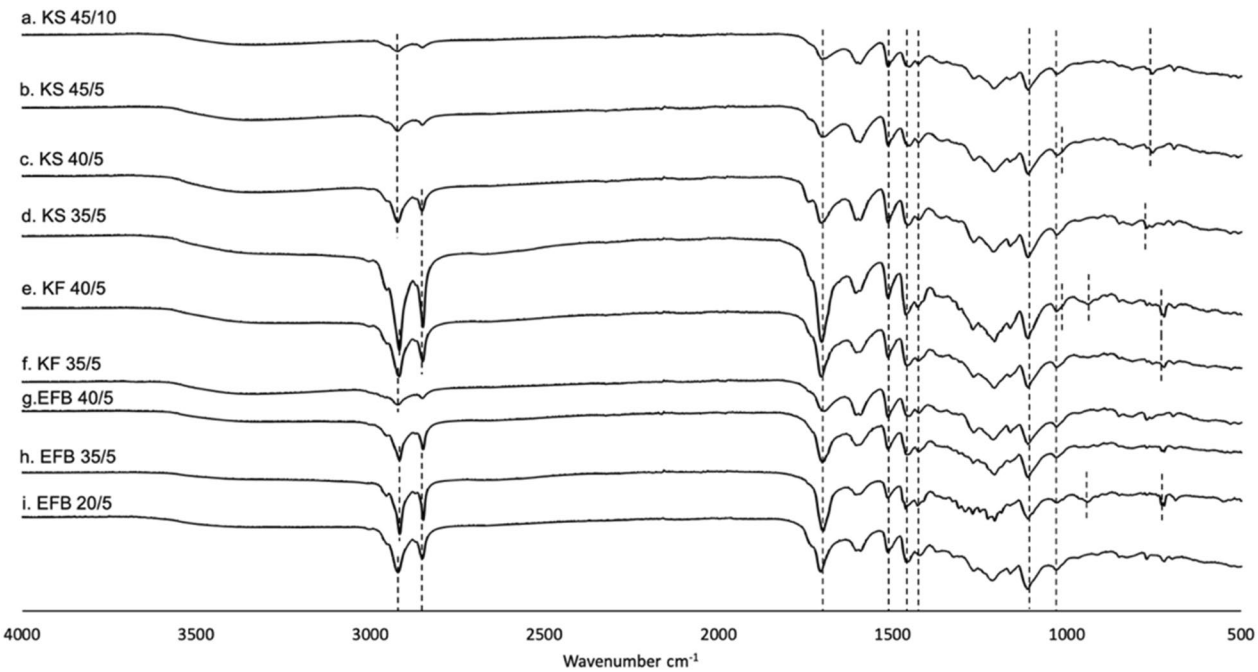


Fig. 4 FT-IR analysis of LML of different steam-exploded (SE) pretreatments (steam pressure/steam time) for kernel shell (KS), kernel fiber (KF), and empty fruit bunches (EFB). **a** KS 45/10, **b** KS 45/5, **c** KS 40/5, **d** KS 35/5, **e** KF 40/5, **f** KF 35/5, **g** EFB 20/5, **h** EFB 35/5, **i** EFB 40/5

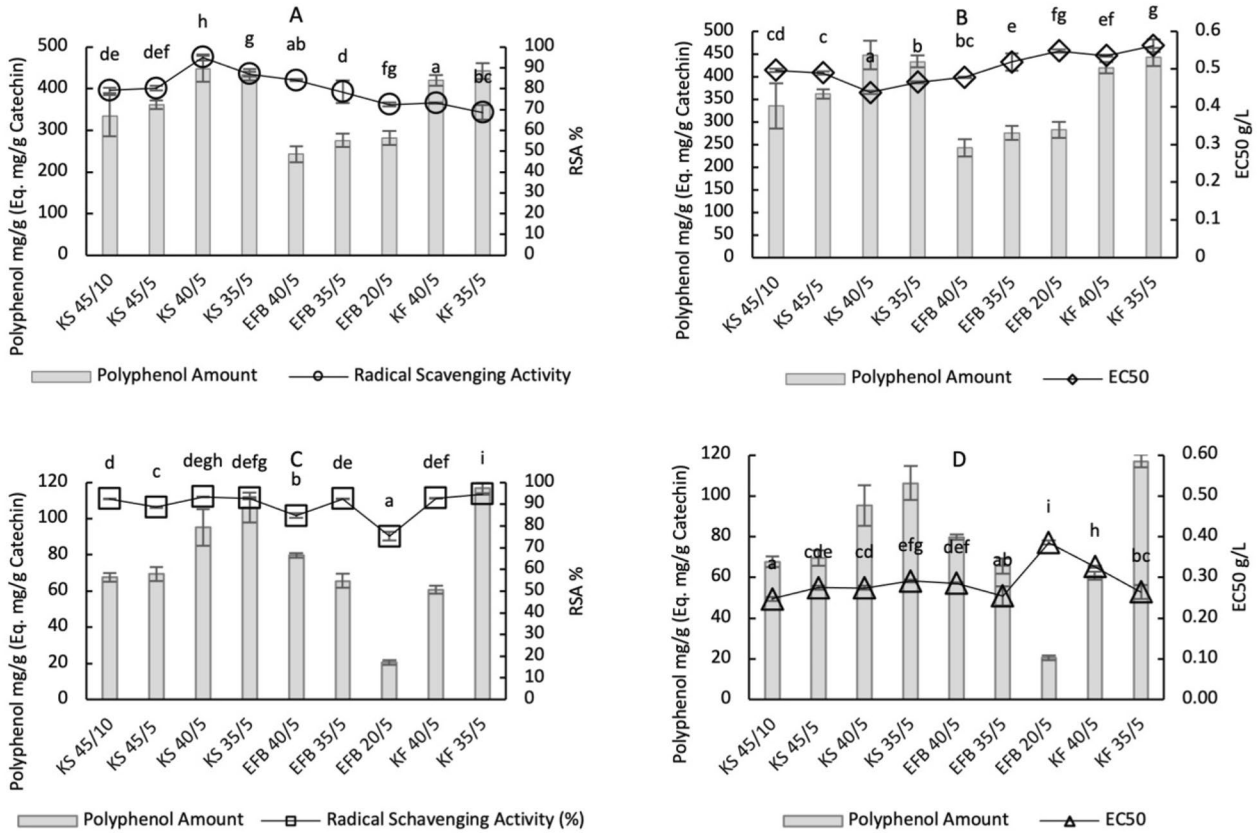


Fig. 5 Antioxidant activity by RSA and EC50 compared to the polyphenol amount. **A** Polyphenol amount from LML compared to its RSA percentage; **B** polyphenol amount from LML compared to the EC50; **C** polyphenol amount from WSL compared to its RSA percentage; **D** polyphenol amount from WSL compared to the EC50. The alphabetic symbol was used as a post hoc range of DMRT 5% to describe the significance of different pretreatment of each biomass

[29–32], which is used for WSL [33, 34]. The abstraction of a hydrogen atom by free radicals, as the rate of the hydrogen atom from the phenyl group of an antiradical substance, becomes a key point in RSA efficiency, which also results in its stability [4]. The half-maximal effective concentration (EC50) is commonly used to measure antioxidant activity via radical scavenging activity; low EC50 values indicate high antioxidant activity [35, 36]. Figure 5 shows the antioxidant activity from radical scavenging using DPPH as free radicals and the polyphenol amount using the Folin–Ciocalteu method to express the polyphenol amount from steam-exploded EFB, KS, and KF of LML and WSL. The polyphenol content was equivalent to the catechin content per sample (mg/g).

A comparison between different severity factors for each POMLBW resulted in different levels of polyphenol production. The lowest severity factor had the lowest molecular weight, influenced by the severity condition, which only cuts a small portion of the lignin from the LCC bonds and produces lignin with a low molecular weight. The increasing severity factor depolymerized a larger portion of the lignin from the LCC, which resulted in more derived lignin with various molecular weights. This phenomenon results in a high average molecular weight. If the severity factor continues to increase, the lignin-derived products continue to depolymerize, resulting in a low average molecular weight. Figure 6 shows the expression of polyphenol production as a function of LML molecular weight. Polyphenol production from KS showed the same trend as that between LML and WSL, where polyphenol production increased at 40/5 and slightly decreased at 45/5 and 45/10, which is similar to the increase in LML molecular weight. EFB was produced in opposite trends between LML and WSL. Increasing the severity factor increased the amount of polyphenol produced in WSL, which was inversely proportional to polyphenol production in LML. In this case, increasing the severity factor decreased the polyphenol content. KF exhibited a similar trend in LML and WSL. The increasing severity factor decreased the polyphenol content, in line with the continuing depolymerization of LCC with an increased average molecular weight of lignin. The

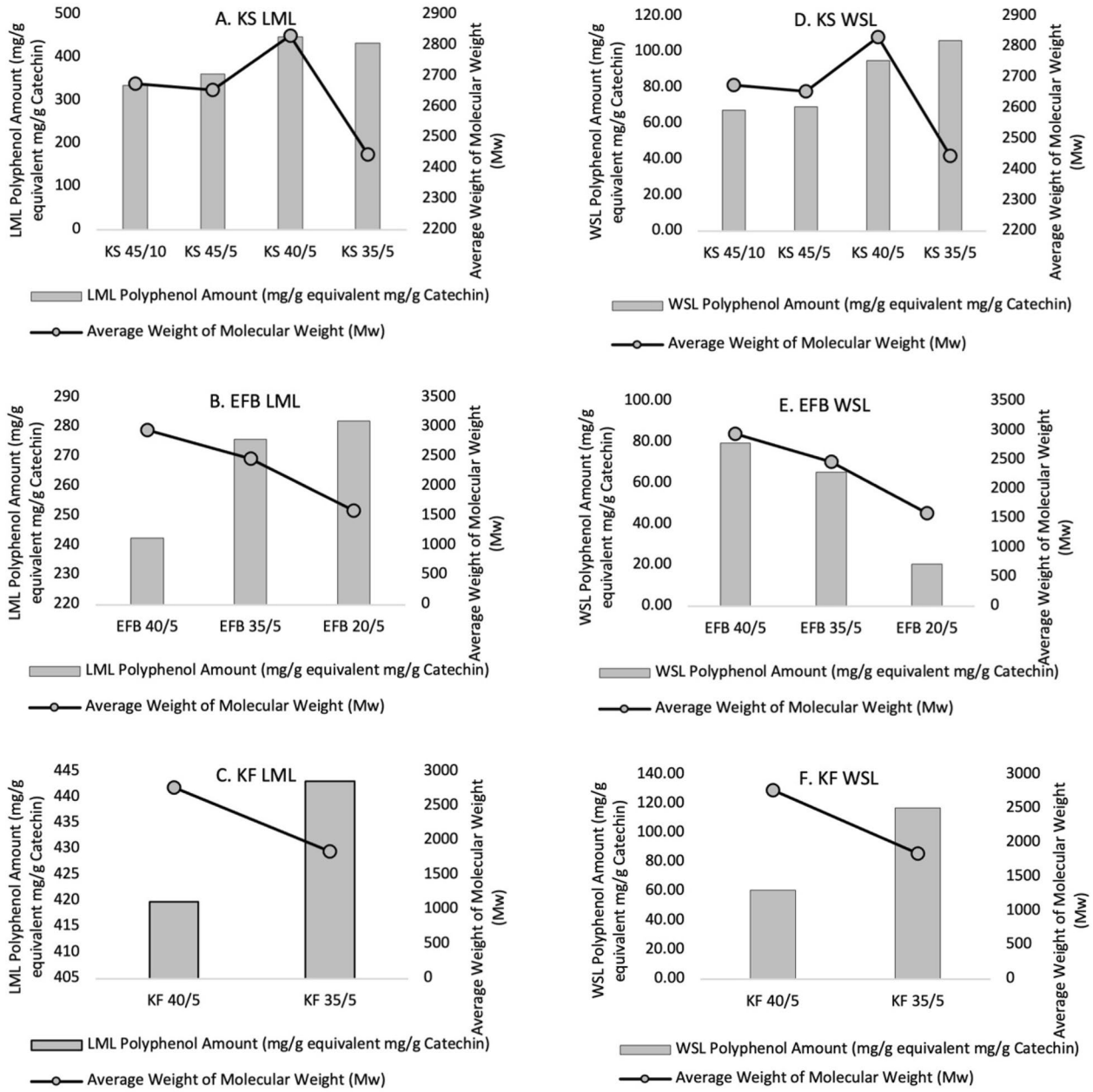


Fig. 6 Expression of polyphenol amount compared to LML molecular weight. **A** KS LML, **B** EFB LML, **C** KF LML, **D** KS WSL, **E** EFB WSL, **F** KF WSL

total polyphenol amount of WSL was 20.47, 65.61, and 79.76 mg/g for 20/5, 35/5, and 40/5 steam-exploded EFB pretreatments, respectively. The differences in polyphenol levels from the WSL in the different pretreatments were caused by the influence of pressure and residence time. EFB 40/5 produced the highest amount of polyphenols and exhibited the lowest EC50 (0.25 g/L). However, the highest RSA value (92.52%) was obtained for KF 35/5, with the lowest EC50 value compared to others. The highest RSA value of the LML steam-exploded EFB was found at 40/5, which also had a low EC50 value. However, polyphenol production was lowest at 242 g/L. The total polyphenol amount in the steam-exploded KF differed at 117.01 and 60.73 mg/g for KF 35/5 and 40/5, respectively. The polyphenol amounts in LML were 282, 276, and 242 from 20/5, 35/5, and 40/5, respectively. Both LML and WSL from steam-exploded EFB exhibited high radical-scavenging activity. In general, for all steam-exploded POMLWB, the EC50 of LML was slightly higher than that of WSL, with a decreasing trend in line with the

increasing severity factor. The WSL from KF 35/5 generated the lowest EC50 (0.26 g/L) and the highest RSA value (94.57%), with 117 and 61 mg/g of polyphenol from 35/5 and 40/5, respectively. The polyphenol amount from LML was 443 and 420 mg/g, indicating no large differences in EC50 value (0.56 and 0.54 g/L) between 35/5 and 40/5, respectively. The RSA value was 72% from 40/5 to. The WSL from KS 35/5, 40/5, 45/5, and 45/10 produced 106.22, 95.19, 69.44, and 67.58 mg/g of polyphenol, respectively. Sample 45/10 exhibited the lowest EC50 value (0.25 g/L). The highest polyphenol content was found at 35/5, and the highest RSA value was 40/5. The LML from all steam-exploded POMLBW produced a high polyphenol content but generally had lower EC50 and RSA values. However, compared to EFB and KF, the RSA value and the EC50 of LML KS were higher with 95% and 0.44 g/L from 40/5, respectively, where the polyphenol amount was 448 mg/g, which was the highest amount compared to other severity factors in LML KS. These results indicate that the highest amount of polyphenol was not always in line with the RSA and EC50 values. Such differences were also reported by Noda et al. [33], who revealed that high levels of polyphenols from WSL did not indicate high RSA levels. Furthermore, the result from the comparison of lignin waste from ethanol production and commercial lignin had similar conditions, which described that the high amount of phenolic compound is not in line with the high oxygen radical absorbance capacity (ORAC) [3]. The antioxidant activity of lignin stems from its abundant phenolic moieties, which participate in proton-coupled electron transfer. Studies have indicated that the enhancement of the antioxidative potential of lignin requires a higher presence of phenolic hydroxyl and methoxyl groups while minimizing the presence of aliphatic oxygen-containing groups such as hydroxyl, carbonyl, and ester groups [7, 29]. Additionally, lignin with a lower molecular weight and narrower polydispersity contributes to stronger antioxidative effects [4]. The low molecular weight of lignin from lignin-derived products of all steam-exploded palm oil lignocellulosic biomass waste resulted in high radical scavenging activity, with approximately 95% from WSL and 80% from LML with 0.25 g/L and 0.5 g/L of EC50 value.

Antimicrobial activities of LML

WSL contains products derived from cellulose and hemicellulose that have antimicrobial activity, such as 5-HMF and furfural [13]. As the antimicrobial activity was concentrated in lignin, we only tested for it in LML. The negative control was N,N-dimethylformamide as a solvent for LML to confirm that it had no antimicrobial activity on any Gram-negative or Gram-positive bacteria. Figure 7 shows the antimicrobial activity of LML against Gram-positive and Gram-negative bacteria. LML in all steam-exploded EFB, KF, and KS inhibited *S. enterica* with an approximately 0.4 cm inhibition zone. Similar results were observed for *E. coli* except for the KS 40/5 pretreatment, where the inhibition zone was only 0.3 cm. LML differed in its inhibition ability for *P. aeruginosa*; an inhibition zone of approximately 0.45 cm was detected for 45/10, 45/5, 40/5 KS, and 35/5 and 20/5 EFB pretreatments. For all KF pretreatments and 40/5 EFB pretreatments, LML created a 0.35 cm inhibition zone for *P. aeruginosa*. The smallest inhibition zone was detected in the KS 35/5 pretreatment. *Staphylococcus aureus*, a Gram-positive, was also inhibited differently by LML in different pretreatments; KF 40/5 and 35/5 resulted in an inhibition zone of 0.4 cm, followed by KS 45/5, 40/5, 35/5, and EFB 35/5. Furthermore, EFB 40/5 and 20/5 resulted in an inhibition zone of approximately 0.32 cm, whereas the smallest inhibition zone (0.16 cm was observed for the KS 45/10 pretreatment.

The high inhibitory activity was suspected to be due to cell lysis, which damaged the cells and leaked the internal fluid. In this process, it was assumed that the polyphenol compounds absorbed reactive oxygen species (ROS), which accumulated on the surface of LML because of their strong antioxidant properties (Fig. 4). This accumulation leads to oxidative stress and disrupts normal redox processes when LML comes in contact with bacteria [15]. Furthermore, owing to their small size, nanoparticles can enter bacterial cells by passing through the cell membrane using a mechanism similar to that of a Trojan horse [4]. During this process, certain monophenolic compounds derived from lignin can enter the bacteria and deplete adenosine triphosphate (ATP) [37] and intracellular pH [38], ultimately leading to cell death. The other reason LML inhibited microbial activity was the scavenging activity of free radicals, such as orthomethoxy groups and the hydroxyl phenolic non-etherified [4], which are contained in the phenolic moieties of LML (Fig. 4). We conclude that all LML produced from steam-exploded POMLBW have antimicrobial properties against both Gram-positive and Gram-negative bacteria. Lignin from EFB has been reported to be antimicrobial against Gram-negative bacteria, such as *E. coli* and *Salmonella typhimurium*, Gram-positive bacteria, such as *Bacillus subtilis* and *S. aureus*, yeasts, such as *Candida albicans*, and fungi, such as *Aspergillus niger* LPB 12 [11]. LML from steam-exploded corn stalks has been reported to have antimicrobial effects against *E. coli* ATCC 25922, *S. enterica* ATCC 9282 as Gram-negative bacteria, *B. subtilis* ATCC 55675, and *S. aureus* CMCC 26003 as Gram-positive bacteria [39]. The lignin fraction has been reported to destroy the cell walls of Gram-positive and

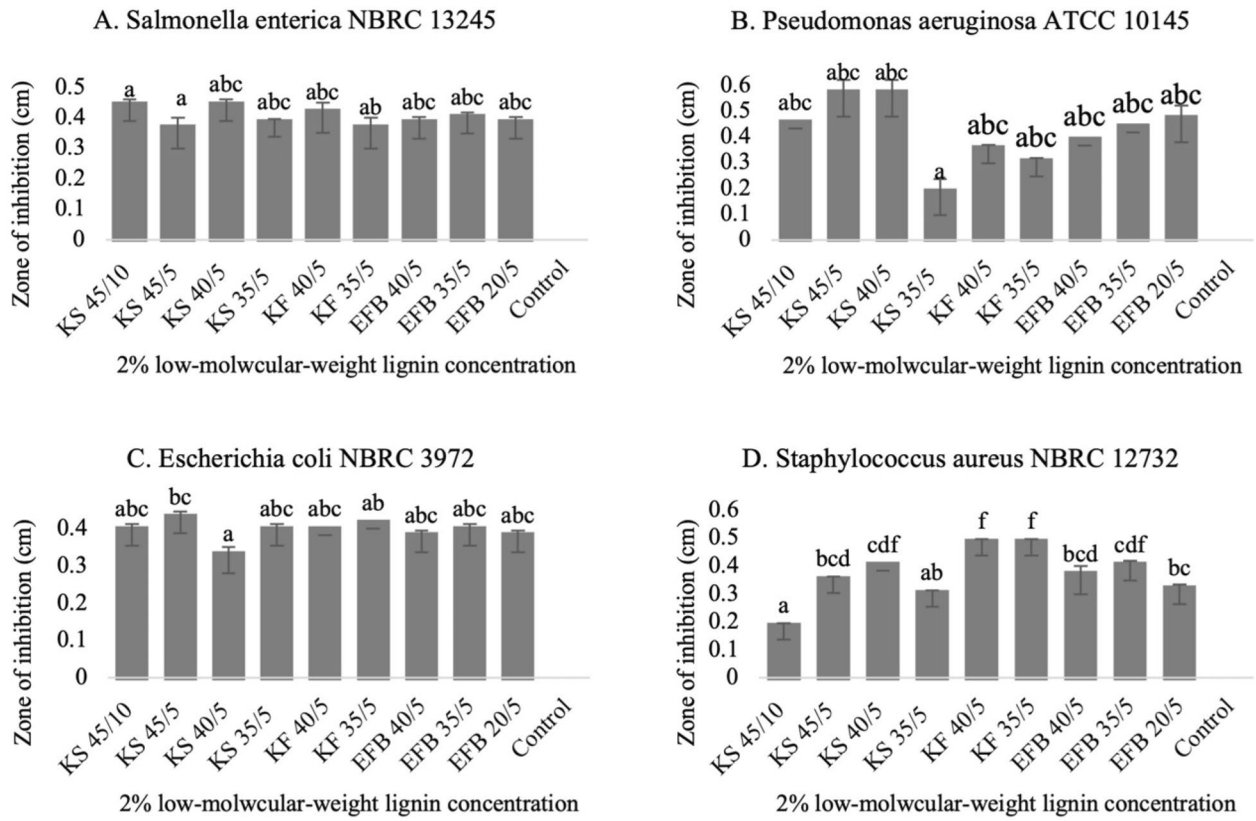


Fig. 7 Antimicrobial activity from Gram-negative **A** *Salmonella enterica*, **B** *Pseudomonas aeruginosa,* **C** *Escherichia coli,* and Gram-positive **D** *Staphylococcus aureus* bacteria using 2% of low-molecular-weight lignin from all steam-exploded POMLBW. The negative control was DMF. The alphabetic symbol was used as a post hoc range of DMRT 5% to describe the significance of different pretreatment of each biomass

Gram-negative bacteria obtained from LML, which have higher phenolic compounds. Furthermore, in vivo examination demonstrated that LML could ameliorate intestinal damage caused by *E. coli-induced diarrhea* [40]. At a concentration of only 2%, LML showed excellent antimicrobial inhibition activity compared to that of lignin nanoparticles (LNP) from acid treatment. It also resulted in a 0.24–0.28 mm inhibition zone for 5% concentration and a 0.33–0.39 mm inhibition zone for 8% for microbial plant pathogens [4]. In another report, phenolic compounds from lignin-derived products could inhibit microbial growth, such as *Aspergillus nigger, Escherichia coli, Bacillus licheniformis*, and *Saccharomyces cerevisiae* by inhibiting enzyme behavior. It is mostly affected by a methyl group and a double bond, which increase the effect of the phenolic compound biocide [41–43]. LML may inhibit microbial enzymes and increase their affinity for cytoplasmic membranes owing to the presence of phenolic hydroxyl groups with high binding affinity, which enhances antimicrobial activity [44–47]. In addition, single hydroxyl group substitution and lipophilicity in the particular degree of the compound increased its antibacterial properties [3, 48, 49]. These reports strengthened this result, where LML from all steam-exploded POMLB contained double bonds and methyl groups (Fig. 4), and all treatments showed various active inhibition abilities for Gram-negative *Salmonella enterica*, *Pseudomonas aeruginosa, Escherichia coli*, and Gram-positive *Staphylococcus aureus*.

Thermal character of LML

Figure 8 shows the glass transition temperatures (T_g) obtained from the DSC analysis of the LML steam-exploded EFB, KF, and KS. The thermal properties of LML can be determined by differential scanning calorimetry (DSC), which is a widely used method for determining lignin or lignin fractions. However, the structural complexity of lignin or lignin-derived products is often difficult to detect, and is mostly detected by curve changes. In EFB 20/5 and 40/5, T_g was recorded at 85 °C; however, the 35/5 pretreatment showed the lowest T_g at 47 °C. The melting temperatures (T_m) of EFB were 362, 343, and 339 °C for 20/5, 35/5, and 40/5, respectively. Compared to lignin, which was sourced from thermal acid hydrolysis (H_2SO_4)

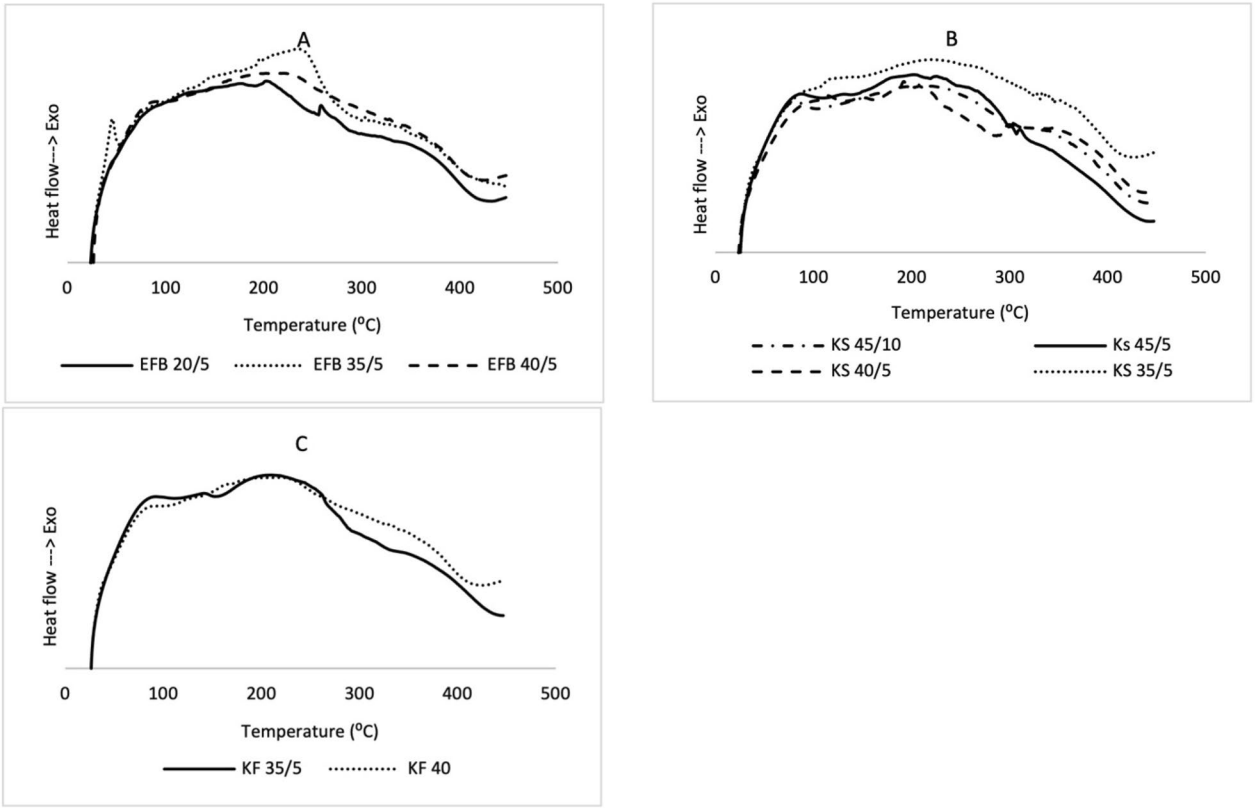


Fig. 8 DSC curve of low-molecular-weight lignin of **A** empty fruit bunches (EFB), **B** kernel shell (KS), and **C** kernel fibers (KF) for different pressure and residence time

and alkaline (NaOH) EFB resulted in a 70 °C glass transition with a melting point of 420 °C [11]. The T_g and T_m from KS resulted in 88, 90, 85, and 80 °C, and 338, 347, and 338 °C from 35/5, 40/5, 45/5, and 45/10, respectively. The T_g from KF was 85 and 80 °C for 35/5 and 40/5, respectively, with the same T_m point at 362 °C. Because LML from all POMLBW under all conditions was included in derivative lignin products, it generated a T_g value that was lower than the normal range for lignin, which normally ranges from 90 to 180 °C [50].

The thermal stability and thermal decomposition of LML were investigated using TG analysis, as shown in Fig. 8, which describes the thermal decomposition (TG) with weight loss temperatures of 5% (T_{d5}), 10% (T_{d10}), and 30% (T_{d30}), with the maximum temperature (T_{max}) included in the remaining residue percentage. The starting temperature of losing mass (T_s), which is used to identify the temperature at which the samples start losing mass, was defined from the weight loss temperatures at 5% and 30% using the following equation: [51, 52]:

$$T_s = 0.49[T_{d5} + 0.6(T_{d30} - T_{d5})].$$

Figure 9A shows the TG from LML EFB 20/5 with 174, 194, and 228 °C for T_{d5}, T_{d10}, and T_{d30}, respectively, with T_{max} 722 °C and 21% residue, where the T_s was 101 °C. LML EFB 35/5 was 177, 194, and 251℃ for T_{d5}, T_{d10}, and $T_{d30,}$ respectively, with T_{max} 722 ℃ and 30% residue, where the T_s was 108 ℃ for 108, and 128 ℃ for 20/5, 35,5, and 40/5, respectively. The conditions for 40/5 were 174, 209, and 318 °C for T_{d5}, T_{d10}, and $T_{d30,}$ respectively, with T_{max} 724 °C and 38% residue, where the T_s was 128 °C. The peak from the LML EFB changed in line with the increased severity factor, which affected the increase in $T_{d30,}$ similar to the percentage of residue. Furthermore, the start temperature (T_s) for the LML EFB 40/5 condition was the highest compared with the other conditions, with an increase of 26.7%. Figure 8B displays LML KS, where the conditions 35/5 resulted in 165, 199, and 302 °C for T_{d5}, T_{d10}, and $T_{d30,}$ respectively. The 40/5 condition resulted in 147, 210, and 312 °C for T_{d5}, T_{d10}, and $T_{d30,}$ respectively. Condition 45/5 resulted in 142, 210, and 312 °C for T_{d5}, T_{d10}, and $T_{d30,}$ respectively. Condition 45/10 resulted in 146, 215, and 322 °C for T_{d5}, T_{d10}, and $T_{d30,}$ respectively. All conditions had a T_{max} of approximately 724 °C, with

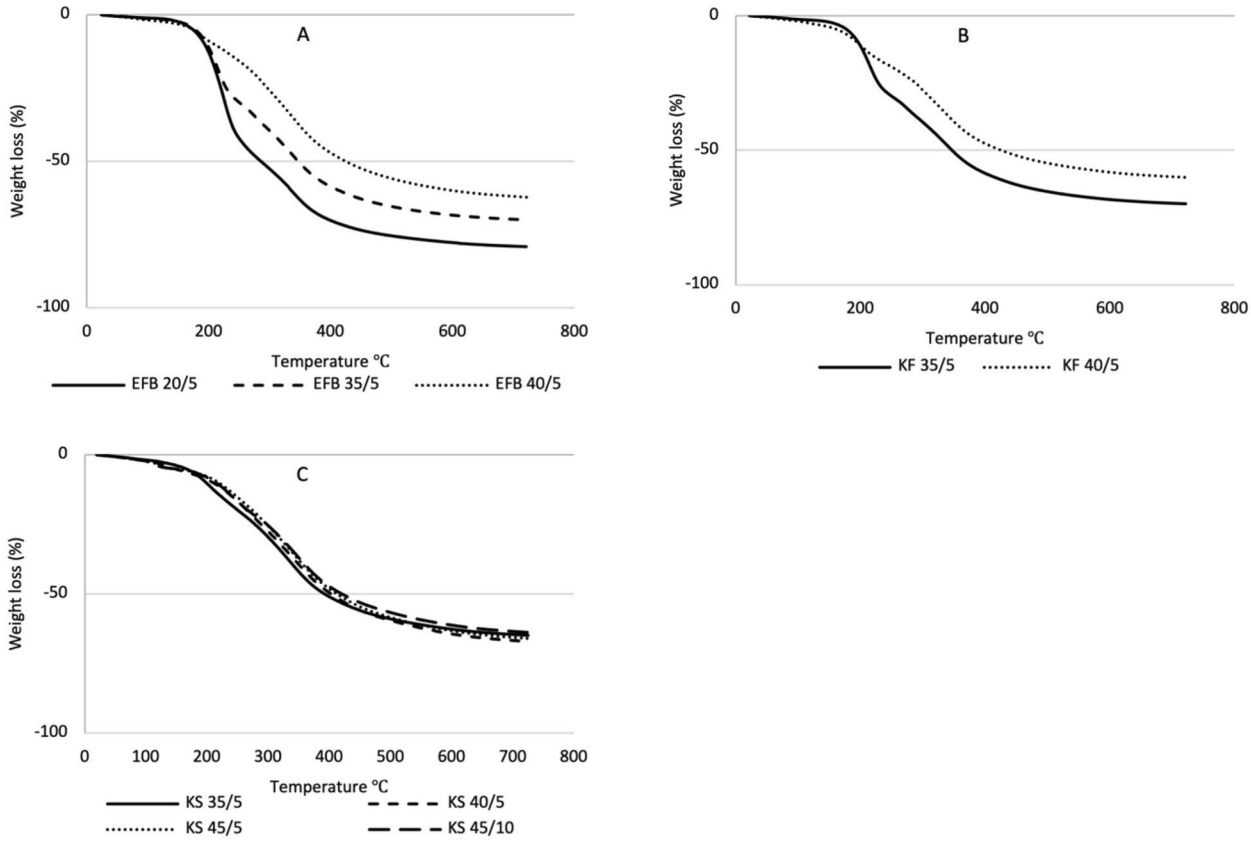


Fig. 9 TG curve of low-molecular-weight lignin of **A** empty fruit bunches, **B** kernel fiber, and **C** kernel shell for different pressure and residence time

33–36% residues where the T_s was 121–123 °C. The LML KF 35/5 resulted in T_{d5}, T_{d10}, and T_{d30} at 177, 197, and 251 °C, respectively, with T_{max} at 722 °C with 30% residue and T_s at 108 °C. Condition 40/5 resulted in T_{d5}, T_{d10}, and T_{d30} at 162, 196, and 310 °C, respectively, with T_{max} at 727 °C with 40% residue, where the T_s was at 123 °C. The thermal characteristics of the LML KF (Fig. 8 C) were similar to those of the EFB, where T_{d30} increased in line with the increasing severity factor, which also caused the increase in T_s (Table 2). The LML KS (Fig. 9C) with a condition higher than 35/5 resulted in the lowest T_{d5} value; however, the values of T_{d10} and $T_{d30,}$ were higher compared to the LML EFB and KF. The T_s and T_{max} from all LML KS were approximately 121 °C and 140 °C, respectively.

Table 2 The LML thermal character

	T_{d5} (℃)	T_{d10} (℃)	T_{d30} (℃)	T_{max} (℃)	Residue (%)	T_s (℃)	T_c (℃)	$T_{melting}$ (℃)
EFB 20/5	174	194	228	722	21	101	105	362
EF 35/5	177	197	251	722	30.05	108	89	343
EFB 40/5	174	209	318	724	37.69	128	102	339
KS 35/5	165	199	302	726	35.20	121	104	338
KS 40/5	147	210	312	724	32.93	121	106	347
KS 45/5	142	219	320	724	34.14	122	110	338
KS 45/10	146	215	322	724	36.28	123	105	344
KF 35/5	177	251	251	722	30.05	108	114	362
KF 40/5	162	196	310	727	39.86	123	115	362

Conclusion

Steam explosion pretreatment of all types of POMLBW resulted in WSL and LML, which had high RSA values, polyphenols, and low EC50 values. The presence of methoxy groups and hydroxyl phenolic groups was suitable for antimicrobial activity in LML from all steam-exploded POMLBW in all conditions, including its molecular size of 1.5–2.8 nm (by conversion). All factors, including antioxidant activity, molecular size, and biocide factor from the presence of hydroxyl phenolic and methoxy groups, correlated with high antimicrobial activities, which strongly suggests a role for oxidative stress and cell infiltration in antimicrobial activity. Furthermore, the thermal behavior of these LMLs is suitable for their application as antimicrobial and antioxidant agents for packaging and other applications that require temperature stability.

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Author contributions
All the authors contributed to the manuscript. SS: material preparation, investigation, data curation, and writing of the original draft. DYA: investigation and data curation. VKN: investigation. CA: review and supervision. YN**:** conceptual review and supervision.

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Declarations

Ethics approval and consent to participate
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Consent for publication
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Competing interests
All authors declare that they have no conflict of interest.

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