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ORIGINAL ARTICLE

The Inhibition of Hepatic and Renal Glucuronidation of *p*-Nitrophenol and 4-Methylumbelliferone by Oil Palm Empty Fruit Bunch Lignin and Its Main Oxidation Compounds

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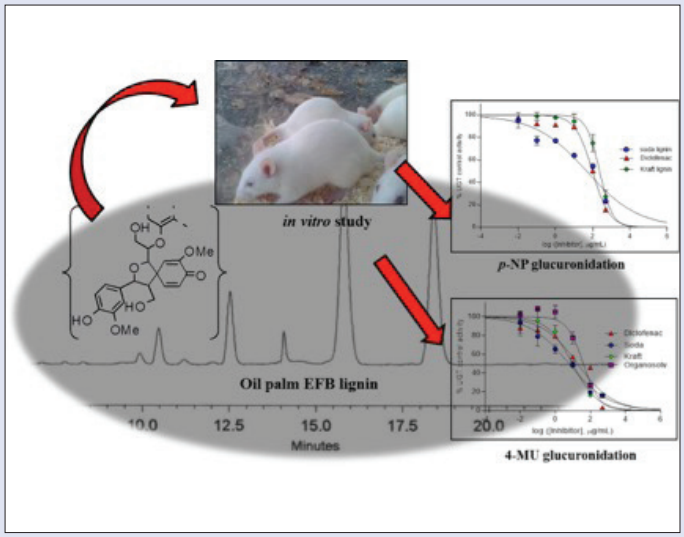
ABSTRACT

Background: In order to develop oil palm empty fruit bunch (EFB) lignin as a nutraceutical and health supplement, the investigation of its potential in interacting with other drugs via inhibition of drug-metabolizing enzymes (DMEs) would ensure product safety. **Objective:** The study was aimed to investigate the *in vitro* effect of oil palm EFB lignin and its main oxidation compounds on phase II DME UDP-glucuronosyltransferases (UGTs) in rat liver and kidney microsomes. **Materials and Methods:** The *p*-nitrophenol (*p*-NP) and 4-methylumbelliferone (4-MU) were employed as probe substrates in glucuronidation assays. The effect of soda oil palm EFB lignin on V_{max}, K_m, CL_{int}, K_i, and mode of inhibition of 4-MU glucuronidation in RLM was also determined. **Results:** The inhibitory potency of oil palm EFB lignin for both *p*-NP and 4-MU glucuronidation in rat liver microsome (RLM) and rat kidneys microsomes (RKM) was found to be in the rank order of soda > kraft > organosolv. However, the inhibitory potency of its main oxidation compounds were in the rank order of vanillin > syringaldehyde > *p*-hydroxybenzaldehyde. Soda oil palm EFB lignin exhibited mixed-type inhibition against 4-MU glucuronidation in RLM, showing the change in apparent V_{max} and with only a minor effect on K_m compared with control. **Conclusions:** The findings showed that effect of oil palm EFB lignin on both *p*-NP and 4-MU glucuronidation in RLM and RKM was enhanced by the presence of vanillin as well as flavonoids. Kinetic study showed that soda oil palm EFB lignin exhibited strong inhibition on UGT activity in RLM with mixed-type inhibition mode.

Key words: *In vitro*, oil palm EFB lignin, 4-MU glucuronidation, *p*-NP glucuronidation, UDP-glucuronosyltransferase, vanillin

SUMMARY

- The inhibitory potential of oil palm EFB lignin extracts for *p*-NP and 4-MU glucuronidation in RLM and RKM can be listed in the following rank order: soda > kraft > organosolv
- The inhibitory potential of oil palm EFB lignin main oxidation compounds for *p*-NP and 4-MU glucuronidation in RLM and RKM can be listed in the following rank order: vanillin > syringaldehyde > *p*-hydroxybenzaldehyde
- Results suggested that the effect of oil palm EFB lignin on *p*-NP and 4-MU glucuronidation activity in both RLM and RKM was enhanced by the presence of vanillin as well as total flavonoid content
- Results also suggested that oil palm EFB lignin may inhibit glucuronidation of substrate by UGT enzymes, especially UGT1A6, particularly in rat liver



Abbreviations used: *p*-NP: *p*-Nitrophenol, 4-MU: 4-Methylumbelliferone, EFB: Empty fruit bunch, DME: Drug-metabolizing enzymes, UGT: UDP-glucuronosyltransferase, V_{max}: Maximal reaction velocity, K_m: Michaelis-Menten constant, CL_{int}: Intrinsic clearance, K_i: Dissociation constant of an inhibitor enzyme complex, 4-MUG: 4-Methylumbelliferone glucuronide, DMSO: Dimethyl sulfoxide, IC_{50}: Half maximal inhibitory concentration, *p*-NPG: *p*-Nitrophenol glucuronide, RKM: Rat kidneys microsomes, RLM: Rat liver microsome, UDPGA: UDP-glucuronic acid, TCA: trichloroacetic acid, MPA: mycophenolic acid

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INTRODUCTION

The renewable resource namely lignocellulosic (waste from oil palm empty fruit bunch [EFB]) as a raw material in the pulp and paper industry has been investigated by many researchers due to its high α-cellulose content.[1,2] Through the alternative and its potential in the future pulp and paper industry, it can decrease the deforestation rate in the country. The alkaline pulping process such as soda and kraft pulping is known as potential pulping process for non-woody materials.[3,4] Both of these pulping processes function to liberate lignin, hemicelluloses, and cellulose to enhance the quality of the pulp.[2]

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The main difference of soda pulping compared with the kraft pulping is the sulfur-free medium of the cooking liquor.[5] Soda lignin is sulfur-free; therefore, its chemical composition is closer to natural lignin compared with kraft lignin.[6,7] While the organic pulping process such as organosolv pulping is also one of the most promising alternatives to existing pulping technologies. Lignin is separated via solubilization from organosolv pulping process. Solubilization makes it possible to obtain a less-modified lignin. The properties of organosolv lignin differ from other technical lignins. The major features are low molecular weight and high chemical purity.

Lignin is a major cell wall component in plants. Lignin provides support for the plant, contributes to the transport of nutrients and water and protects against microorganisms.[8] Lignin is an amorphous polymer built up by oxidative coupling of three major phenylpropanoid units namely trans-*p*-coumaryl alcohol, trans-coniferyl alcohol, and trans-sinapyl alcohol. The phenylpropanoids form a randomized structure in a three-dimensional network inside the cell wall. The phenylpropane units are commonly classified into three main types that differ in the amount of methoxyl groups, namely *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin.[9] These basic units are primarily attached to different types of aryl-aryl ether type and have more than 10 different types of linkages.[10]

Besides producing biomaterials products, lignin that can be extracted from any lignocellulosic waste such as oil palm EFB, sugar cane, wood and others, has potential use in pharmaceutical and food product industry such as emulsifier,[11] antidiarrheal drug,[12] and natural antioxidant.[13-16] The development of oil palm EFB lignin for its application in pharmaceuticals and food industries promises an additional value to lignin. However, before oil palm EFB lignin can be developed as a nutraceutical and health supplement, the investigation of its potential in interacting with other drugs or pharmaceutical agents must be carried out to ensure product safety. This is because many dietary polyphenolic compounds undergo extensive biotransformation including oxidation, reduction, methylation, sulfation, glucuronidation, and microbial degradation.[17,18] Therefore, oil palm EFB lignin, which is composed of polyphenol compounds, would have the potential to be metabolized by the same drug-metabolizing enzymes (DMEs) as other drugs or pharmaceutical agents. Therefore, it could result in food-drug interaction and may cause adverse side effects.[19-21]

DMEs are a diverse group of proteins that are responsible for metabolizing a vast array of xenobiotic chemicals, including drugs, carcinogens, pesticides, pollutants and food toxicants, as well as endogenous compounds such as steroids, prostaglandins and bile acids.[22-24] Generally, DMEs eliminate foreign compounds (xenobiotics) and endogenous substances by increasing solubility through the functionalization processes in phase I and/or conjugation reactions in phase II.

The glucuronidation reaction is the most important detoxification pathway of the phase II drug metabolism in human and animals. Conjugation of suitable functional group present on a substrate with UDP-glucuronic acid (UDPGA) as a cofactor and catalyzed by the enzyme UDP-glucuronosyltransferase (UGT) is responsible for the elimination pathway of many xenobiotics such as drugs, chemical carcinogens, and endogenous compounds such as bilirubin, steroid hormones, and fat-soluble vitamins in humans and animals.[25,26] In addition, glucuronidation facilitates excretion of these compounds and also the products of phase I metabolism as their hydrophilic conjugates in urine and bile, although a limited number of glucuronides possess biological activity.[27]

Given the importance of glucuronidation conjugation as a detoxification and metabolic pathway for numerous xenobiotics and endogenous compounds and the development of oil palm EFB lignin for pharmaceuticals and food industries applications, there is growing interest in the study of the potential for its interaction effect on UGT enzymes activity. Therefore, the main aim of this study was to determine the *in vitro* effect of oil palm EFB lignin (soda, kraft, and organosolv) and its main oxidation compounds (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde) on UGT enzymes activity in rats by using *p*-nitrophenol (*p*-NP) and 4-methylumbelliferone (4-MU) as probe substrates. 4-MU and *p*-NP are known to be metabolized by multiple UGT isoforms and also represent convenient probes for comparing the glucuronidation activity and kinetics of tissues and recombinant enzymes. Rat liver microsomes (RLM) and rat kidneys microsomes (RKM) were used as sources of the UGT enzymes. Liver is known as the major "metabolic clearing house" for both endogenous and xenobiotics compounds. However, UGT enzymes activity also appears to have a distinct pattern of extrahepatic expression, particularly in the kidney.[28] Therefore, it is especially necessary to examine the effect of oil palm EFB lignin on both hepatic and renal UGT enzymes activity in rats.

This study was carried out to determine the IC_{50} values for oil palm EFB lignin and its main oxidation compounds on liver and kidney UGTs activity in rats by *in vitro* study. Further, the maximal velocity of reaction (V_{max}), Michaelis-Menten constant (K_m) value, intrinsic clearance (CL_{int}), inhibition constant (K_i) and mode inhibition in an inhibitor concentration-dependent were determined for the inhibitor that showed the lowest IC_{50} value among all oil palm EFB lignin.

MATERIALS AND METHODS

Materials

The raw material used in this study consisted of oil palm EFB 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 h at a maximum cooking temperature of 170°C. The ratio of cooking liquor to EFB fiber was 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 25% of sulfidity with water-to-fiber ratio of 8. The digester was heated from room temperature to 170°C for 1 h and continued at 170°C for 2 h. For soda pulping, 25% of active alkali with no percentage of sulfidity was applied under similar conditions. For organosolv pulping pretreatment process, a 1.0-L glass-lined pressure Parr reactor with Parr 4842 temperature controller (Parr Instrument Company, Moline, IL) was employed. A mass of 22.0 g of EFB (dry mass) was treated with ethanol/water mixture in a volume ratio of 65:35 with sulfuric acid as the catalyst.[29] The solid-to-liquid ratio used was 1:8. The reactor was heated at room temperature to 190°C for 1 h and continued at 190°C for 45 min.

Preparation of oil palm EFB 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 h.[30] The preparation of organosolv OPEFB lignin was carried out by washing the pretreated 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 oil palm EFB lignin

The lignin sample was purified by extracting it in the soxhlet apparatus for 6 h 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 nonlignin phenolic compounds that may remain in the black liquor. The purified lignin was then dried further in the oven at 45°C for another 24 h before storage in plastic bottles for further analysis.

Animal sources

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-h light/12-h dark conditions for 1 week before the start of the experiments. They were provided with water and food *ad libitum*. 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 liver and kidneys were taken out for microsomes preparation.

Preparation of rat liver and kidneys microsomes

Microsomes were prepared as described by Gibson and Skett.[31] Rat livers and kidneys were removed immediately after sacrifice and 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 min at 4°C, the resultant supernatant was decanted to ultracentrifuge tubes (Optiseal™) and centrifuged at 100,000*g* for 60 min in a Optima™ TLX refrigerated ultracentrifuge (Beckman Coulter, Inc., USA). The supernatant contains microsomal and soluble cytosolic fraction. The microsomal pellet obtained was reconstituted and each was resuspended in 300 µL of 67 mM potassium phosphate buffer with 1.15% (w/v) KCl and 20% (v/v) glycerol. The pooled microsomes were homogenated again to mix the solution. The supernatants were kept at −80°C until use.

Determination of protein content

Protein concentration of the RLM and RKM were determined by Lowry's method using bovine serum albumin (BSA) as the standard,[32] with slight modification according to Pomory.[33] The determination of protein content was performed in triplicates.

Kinetic analysis for *p*-NP glucuronidation assay

Substrate saturation experiments were conducted to generate the rate of reaction for determining the apparent substrate K_m and V_{max} for RLM and RKM assay. The K_m and V_{max} values were determined by nonlinear regression analysis of the *p*-NP-glucuronide formation data using seven different substrate (*p*-NP) concentrations (0-3,000 µM). Data points were fitted to the Michaelis-Menten model using Prism 6.0 (Graphpad Software, San Diego, CA).

Inhibition of *p*-NP glucuronidation assay

The method for inhibition of *p*-NP glucuronidation assay was obtained from the work published by Bock *et al.*,[34] with modification by Azizi *et al.*,[35] and was optimized for the current study. The incubation conditions were optimized with respect to time of incubation, microsomal protein concentration, and Triton X-100 concentration. Briefly, the incubation mixture in a total volume of 0.20 mL containing 5 mg mL^{-1} RLM or RKM was pre-incubated with 0.25 v/v % Triton X-100 (0.125 v/v % Triton X-100 for RKM assay) on ice for 5 min. A volume of 20 µL of 1 M Tris-HCl (pH 7.4), 20 µL of 50 mM $MgCl_2$, and 20 µL of 5 mM *p*-NP were added to the mixture. The volume was made up to 0.2 mL with distilled water. The reaction was initiated by adding 20 µL of 30 mM UDPGA. The incubation was carried out in a shaking water bath (Stuart® SBS40) at 37°C for 30 min for both RLM and RKM assays.

The reaction was terminated with the addition of 200 µL trichloroacetic acid (TCA) (20 v/v %). The mixture was vortexed vigorously for 2 min and 200 µL of aliquots in each tube was taken out into a new tube. Then, 0.5 M of NaOH was added and left for 10 min. Absorbance was measured at 405 nm using a microplate reader (Plate CHAMELEON™ multitechnology plate reader, 425-106). Screening experiments were conducted to generate IC_{50} values by incubating *p*-NP at the estimated K_m value in the presence of various concentrations of positive inhibitor (diclofenac), oil palm EFB lignin (soda, kraft, and organosolv) and its main oxidation compounds (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde).

The amount of the substrates glucuronidated was calculated by subtracting the known concentration of *p*-NP in a blank tube with the concentration of the unreacted *p*-NP in the reaction tubes. Consequently, the value of the glucuronidated substrate was divided with the protein concentration used and the incubation time. The equation is illustrated below:

Kinetic analysis for 4-MU glucuronidation assay

The K_m and V_{max} values were determined by nonlinear regression analysis of the 4MU-glucuronide (4-MUG) formation data using different substrate (4-MU) concentrations (0-10 mM). Data points were fitted to the Michaelis-Menten model using Prism 6.0 (Graphpad Software).

Inhibition of 4-MU glucuronidation assay

The method for inhibition of 4-MU glucuronidation assay was adopted from the work published by Hanioka *et al.*[36] and optimized for the current study. The incubation conditions were optimized with respect to time of incubation, microsomal protein concentration, and Triton X-100 concentration. The incubation mixture containing a total volume of 0.25 mL (0.1 mg mL^{-1} for liver or 0.125 mg mL^{-1} for kidney) microsomal protein was pre-incubated with Triton X-100 (0.1 µL mg^{-1} protein for RLM or 0.2 µL mg^{-1} protein for RKM) on ice for 5 min. Then, 5 µL of 2.5 M Tris-HCl (pH 7.4), 10 µL of 250 mM $MgCl_2$, and 12.5 µL of 2 mM 4-MU were added into the incubation mixture. The volume was increased to 0.25 mL with distilled water. Control group was replaced with equivalent volume of distilled water instead of test inhibitor samples. After pre-incubation for 5 minutes in a shaking water bath (Stuart® SBS40) at 37°C, the reaction was initiated by adding 12.5 µL of 100 mM UDPGA and the mixture was further incubated in a shaking bath at 37°C for 15 min for both RLM and RKM assays.

Screening experiments were conducted to generate IC_{50} values by incubating 4-MU at the estimated K_m value in the presence of various concentrations of positive inhibitor (diclofenac), potential inhibitors, oil palm EFB lignin (soda, kraft, and organosolv), and its main oxidation compounds (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde). The reaction was terminated with the addition of 100 µL TCA (20 v/v %). The mixture was vortexed and placed on ice for 10 min and centrifuged at 2,000 rpm at 4°C for 10 min in order to coagulate the protein. The supernatant fraction (10 µL) was injected into the high-performance liquid chromatography (HPLC) system according to the method that has previously been validated.[37]

The metabolite formed were quantified using a calibration curve of 4-MUG. The concentration of 4-MUG was divided with incubation time and protein concentration used to obtain the UGT-specific activity. The UGT-specific activity was calculated using equation

Further, the inhibition constant (K_i) was determined for the inhibitor with the lowest IC_{50} value among all oil palm EFB lignin extracts. In such cases, concentration of 4-MU (0.1-10 mM) with RLM and a range of concentrations of soda oil palm EFB lignin (0. 2.5, 5.0, and 10 μg mL^{-1}) were used for the construction of Dixon plots and estimation of K_i values.

Determination of 4-MUG

HPLC determination of 4-MUG was performed using a method by Lewis *et al.*,[37] with a slight modification. The method was validated earlier by Haron.[38] Ten microliter of the filtrate was injected into the HPLC system (Shimadzu) equipped with reversed-phase Gemini NX C18 column (particle size 5 μm, 150 mm × 4.6 mm i.d.). The column was kept at 37°C. The eluent was monitored with a UV detector at 316 nm with gradient elution at a flow rate of 1 mL min^{-1}. The 4-MUG standard solutions were freshly prepared for each experiment with concentration ranges of 5-100 μM.

RESULTS

The *p*NP-glucuronide and 4-MUG formations in RLM and RKM were best explained by Michaelis-Menten kinetics. For *p*-NP glucuronidation, the K_m and V_{max} values were 860.50 ± 86.81 μM and 43.04 ± 1.64 nmol min^{-1} mg^{-1}, respectively, for RLM and 857.30 ± 68.30 μM and 9.320 ± 1.11 nmol min^{-1} mg^{-1}, respectively, for RKM.

For 4-MU glucuronidation, the K_m and V_{max} values were 0.104 ± 0.020 mM and 27.87 ± 1.08 nmol min^{-1} mg^{-1}, respectively, for RLM and 0.101 ± 0.006 mM and 12.01 ± 0.12 nmol min^{-1} mg^{-1}, respectively, for RKM. Further, the inhibition effects of positive inhibitor (diclofenac), oil palm EFB lignin, and its main oxidation compounds on *p*-NP and 4-MU glucuronidation activity assays by RLM and RKM were performed at *p*-NP and 4-MU concentrations that are close to the apparent K_m value.

The effect of oil palm EFB lignin and its main oxidation compounds on *p*-NP glucuronidation

The inhibitory effects of soda, kraft, and organosolv oil palm EFB lignin on *p*-NP glucuronidation in RLM were screened and the half maximal inhibitory concentration (IC_{50}), which is the effective concentration of soda, kraft, and organosolv oil palm EFB lignin required to decrease UGT control specific activity to 50%, was compared with positive inhibitor (diclofenac) in RLM and is presented in Figure 1.

For all *p*-NP glucuronidation studies, diclofenac was chosen as the positive inhibitor. Diclofenac is a comparatively potent UGT inhibitor for all UGT isoforms.[39] Therefore, diclofenac was employed in this study as a positive inhibitor and showed a good inhibition of *p*-NP glucuronidation in RLM with an IC_{50} value of 245.900 ± 1.195 μM (78.228 ± 0.380 μg mL^{-1}) [Figure 1 and Table 2]. This IC_{50} value obtained was lower than the reported IC_{50} value (411.990 μM) by Azizi *et al.*[35] In RLM, diclofenac inhibited *p*-NP glucuronidation with an IC_{50} value of 821.200 ± 1.094 μM (261.248 ± 0.348 μg mL^{-1}).

Soda oil palm EFB lignin started to show significant inhibition ($P < 0.05$) by RLM at concentration of 0.01 μg mL^{-1} onward, kraft oil palm EFB lignin started to show significant inhibition ($P < 0.05$) at concentration of 1.0 μg mL^{-1} onward, and organosolv oil palm EFB lignin started to show significant inhibition ($P < 0.05$) at concentration of 0.1 μg mL^{-1} onward. The other concentrations showed some changes in *p*-NP glucuronidation activity, but the changes were not statistically significant.

The effect of oil palm EFB lignin on *p*-NP glucuronidation in RKM was also screened and the IC_{50} values for soda, kraft, and organosolv oil palm EFB lignin were compared with positive inhibitor (diclofenac) in RKM and are presented in Figure 2. Results showed that soda, kraft, and organosolv oil palm EFB lignin started to show significant inhibition ($P < 0.05$) by RKM at concentration of 10.0 μg mL^{-1} onward.

In order to examine the contribution of oxidation compounds of oil palm EFB lignin on UGT enzymes activity, the IC_{50} values of three main oxidation compounds (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde) were subsequently determined. The concentration of each oxidation compound in different types of oil palm EFB lignin has been investigated previously.[40] The study has identified and quantified eight compounds from alkaline nitrobenzene oxidation process of oil palm EFB lignin. Among them, syringaldehyde was found have the highest percentage in all three types of oil palm EFB lignin, followed by vanillin and *p*-hydroxybenzaldehyde [Table 1].

The calculated IC_{50} values of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde were compared with that of positive inhibitor (diclofenac) for RLM and RKM [Figures 3 and Figure 4]. An accurate IC_{50} value for *p*-hydroxybenzaldehyde could not be determined because maximum inhibition (>50% inhibition) did not occur at the highest dose investigated.

Vanillin and syringaldehyde started to show significant inhibition ($P < 0.05$) for RLM at concentration of 0.01 μM onward, while, *p*-hydroxybenzaldehyde started to show significant inhibition ($P < 0.05$) at concentration of 1.0 μg mL^{-1} onward. In RKM, vanillin started to show significant inhibition ($P < 0.05$) at concentration of 100.0 μM onward and syringaldehyde started to show significant inhibition ($P < 0.05$) at concentration of 10.0 μM onward.

The effect of oil palm EFB lignin and its main oxidation compounds on 4-MU glucuronidation

The formation of 4-MUG generated by the incubation of 4-MU with RLM was investigated in the presence of oil palm EFB lignin (soda, kraft, and organosolv) and the calculated IC_{50} values of the three types

Table 1: The yield (% dry sample, w/w) of main compounds from alkaline nitrobenzene oxidation of oil palm EFB lignin[39]

Compound	% weight (dry basis)		
	Soda	Kraft	Organosolv
Syringaldehyde	7.074 ± 0.126	5.109 ± 0.007	5.803 ± 0.281
Vanillin	3.334 ± 0.042	2.553 ± 0.037	2.840 ± 0.143
p-hydroxybenzaldehyde	0.140 ± 0.012	0.084 ± 0.039	0.143 ± 0.143

Results are expressed as mean ± SD ($n = 3$).

Table 2: Inhibition of *p*-nitrophenol glucuronidation in RLM and RKM by oil palm EFB lignin and its main oxidation compounds. Data are expressed as the best-fit IC_{50} values ± SD for five replicates (n=5).

Compound	IC_{50} values	
	RLM	RKM
	μg mL^{-1}	μg mL^{-1}
Diclofenac	78.228 ± 0.380	261.248 ± 0.348
Soda oil palm EFB lignin	84.750 ± 1.218	128.400 ± 1.116
Kraft oil palm EFB lignin	232.900 ± 1.068	260.200 ± 1.117
Organosolv oil palm EFB lignin	659.400 ± 1.257	792.400 ± 1.241
	μM	μM
Diclofenac	245.900 ± 1.195	821.200 ± 1.094
Vanillin	60.410 ± 1.164	243.300 ± 1.143
Syringaldehyde	141.900 ± 1.072	380.600 ± 1.102
p-hydroxybenzaldehyde	542.700 ± 1.153	> 1000

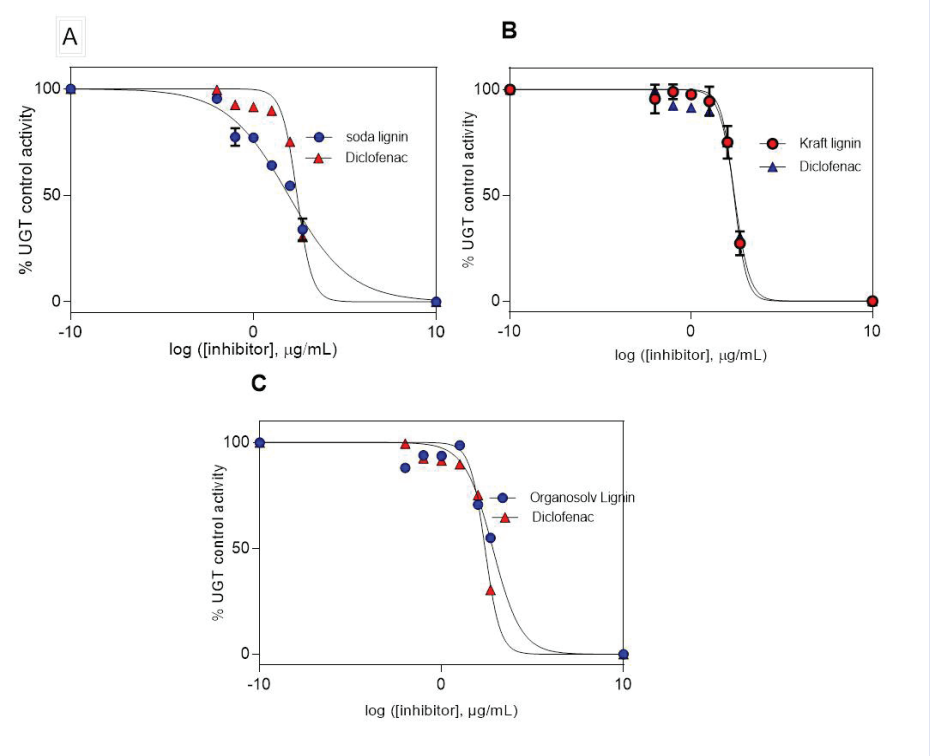

Figure 1: Inhibition of *p*-NP glucuronidation in RML by (a) soda, (b) kraft (c), and organosolv oil palm EFB lignin compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for five replicate incubations (n = 5). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

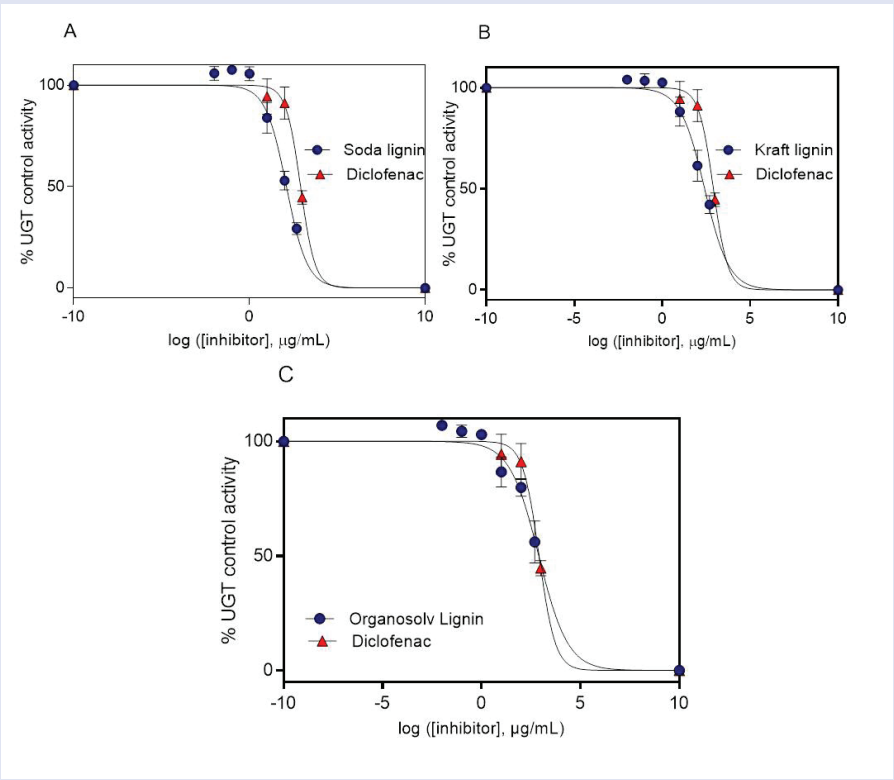

Figure 2: Inhibition of *p*-NP glucuronidation in RKM by (a) soda, (b) kraft, and (c) organosolv oil palm EFB lignin compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for five replicate incubations (n = 5). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

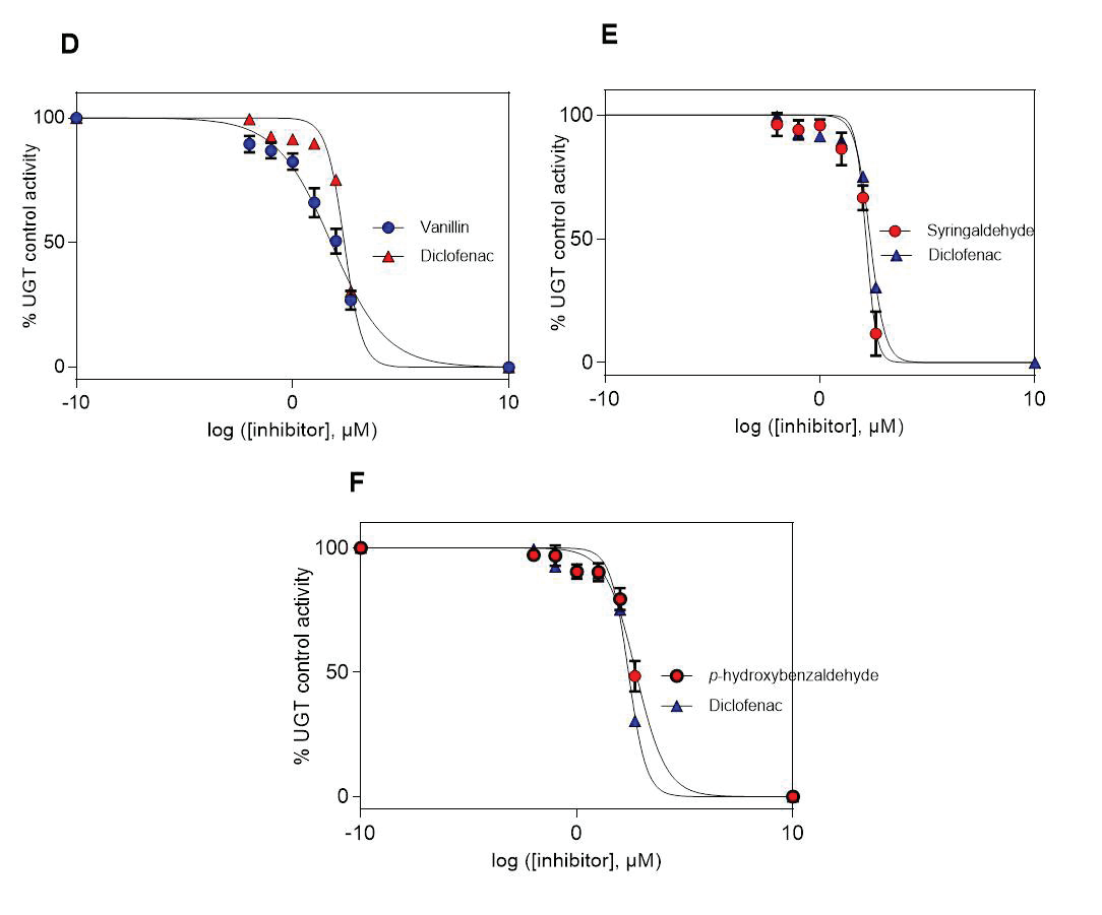

Figure 3: Inhibition of *p*-NP glucuronidation in RLM by (a) vanillin, (b) syringaldehyde, and (c) *p*-hydroxybenzaldehyde compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for five replicate incubations ($n = 5$). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

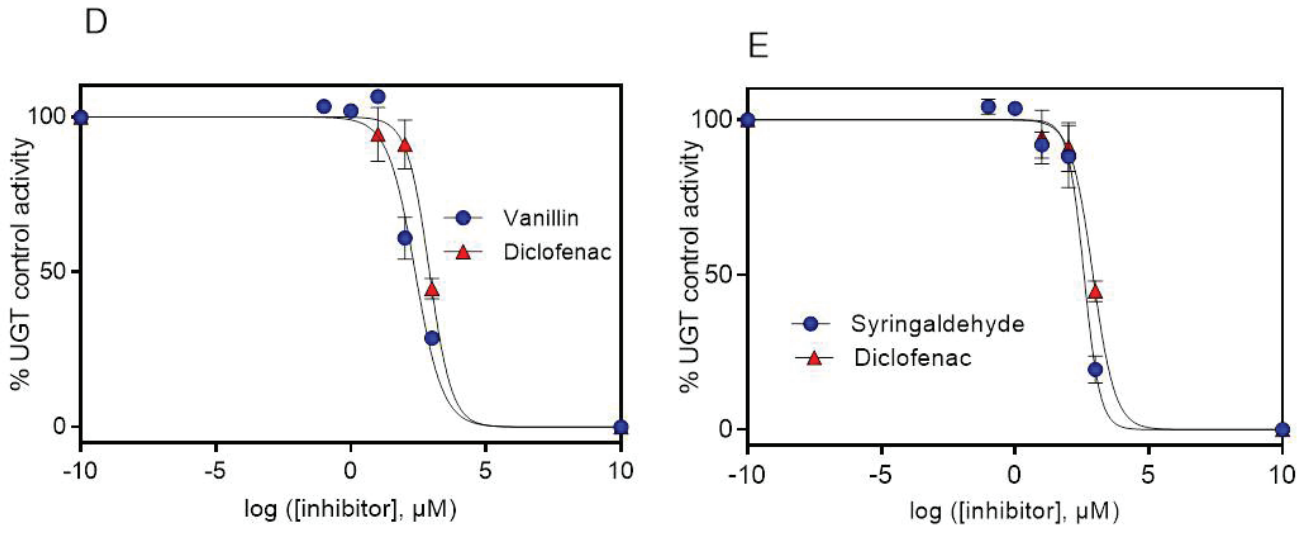

Figure 4: Inhibition of *p*-NP glucuronidation in RKM by (a) vanillin and (b) syringaldehyde compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for five replicate incubations ($n = 5$). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

Table 3: Inhibition of 4-methylumbelliferone glucuronidation in RLM and RKM by oil palm EFB lignin and its main oxidation compounds. Data are expressed as the best-fit IC_{50} values ± SD for three replicates (n=3).

Compound	IC_{50} values	
	RLM	RKM
	μg mL^{-1}	μg mL^{-1}
Diclofenac	15.520 ± 1.627	33.480 ± 1.715
Soda oil palm EFB lignin	6.018 ± 1.231	11.400 ± 1.429
Kraft oil palm EFB lignin	9.558 ± 1.225	29.310 ± 1.161
Organosolv oil palm EFB lignin	40.20 ± 1.150	46.360 ± 1.122
	μM	μM
Diclofenac	48.785 ± 5.114	105.240 ± 5.391
Vanillin	6.121 ± 1.611	16.100 ± 1.754
Syringaldehyde	742.300 ± 1.123	898.700 ± 1.217
p-hydroxybenzaldehyde	> 1000	> 1000

Table 4: Kinetics parameters for 4-MU glucuronidation in concentration-dependents of soda oil palm EFB lignin. Data are expressed as the values ± SD for three replicates (n=3). The statistical analysis was conducted using one-way ANOVA and Dunnet test. *p < 0.05 vs. control (0 μg mL^{-1} of soda oil palm EFB lignin).

Soda oil palm EFB lignin concentration (μg mL^{-1})	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	CL_{int} (μL min^{-1} mg^{-1})
0	0.104 ± 0.020	27.870 ± 1.075	269.464 ± 2.887
2.5	0.160 ± 0.026*	19.110 ± 0.741*	119.551 ± 13.546*
5.0		15.920 ± 0.660*	
10.0	0.122 ± 0.026*	13.150 ± 0.396*	96.516 ± 16.602*
	0.149 ± 0.020*		87.968 ± 2.814*
K_i value (μg mL^{-1})	7.550 ± 0.780 (mixed type)		

of oil palm EFB lignin were compared with that of positive inhibitor (diclofenac) in RLM, as represented in Figure 5.

Diclofenac was also chosen as a positive inhibitor for 4-MU glucuronidation study. A summary of the IC_{50} results presented in Table 3 illustrates the differential effects of oil palm EFB lignin on 4-MU glucuronidation in RLM and RKM compared with diclofenac. Based on this result, diclofenac inhibited 4-MU glucuronidation in RLM with IC_{50} values of 21.14 ± 1.32 μM, 1.6 times more potent than in RKM (IC_{50} values of 33.48 ± 4.15 μM).

Soda oil palm EFB lignin started to show significant inhibition ($P < 0.05$) by RLM at concentration of 0.1 μg mL^{-1} onward, kraft oil palm EFB lignin started to show significant inhibition ($P < 0.05$) at concentration of 1.0 μg mL^{-1} onward, and organosolv oil palm EFB lignin started to show significant inhibition ($P < 0.05$) at concentration of 10 μg mL^{-1} onward. Figure 5 and Table 3 show that the most potent inhibitor for 4-MU glucuronidation in RLM was soda oil palm EFB lignin, followed by kraft and organosolv oil palm EFB lignin (soda > kraft > organosolv).

Meanwhile, the effect of oil palm EFB lignin on 4-MU glucuronidation in RKM was also investigated and the calculated IC_{50} values of oil palm EFB lignin compared with that of positive inhibitor (diclofenac) in RKM are presented in Figure 6 and summarized in Table 3. Soda, kraft, and organosolv oil palm EFB lignin started to show significant inhibition ($P < 0.05$) by RKM at concentrations of 10 μg mL^{-1} onward. Figure 6 and Table 3 show that the most potent inhibitor for 4-MU glucuronidation in RKM was soda oil palm EFB lignin, followed by kraft and organosolv (soda > kraft > organosolv).

Further, the formation of 4-MUG generated by the incubation of 4-MU in RLM and RKM was also investigated in the presence of three main oxidation compounds in oil palm EFB lignin (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde) and the calculated IC_{50} values compared with positive inhibitor (diclofenac) in RLM and RKM are presented in Figure 7 and Figure 8.

As shown in Figure 7, vanillin and syringaldehyde started to show significant inhibition ($P < 0.05$) by RLM at concentration of 0.1 μM onward. While, *p*-hydroxybenzaldehyde started to show significant inhibition ($P < 0.05$) by RLM at concentration of 1.0 μM onward. The same observation was also found for RKM [Figure 8]. Vanillin and syringaldehyde started to show significant inhibition ($P < 0.05$) by RKM at concentration of 0.1 μM onward, while *p*-hydroxybenzaldehyde started to show significant inhibition ($P < 0.05$) by RKM at concentration of 1.0 μM onward.

The effect of soda oil palm efb lignin on michaelis-menten kinetics of 4-mu glucuronidation in rlm

Since soda oil palm EFB lignin showed the most potent inhibition for UGT activity [Table 2 and Table 3], the *in vitro* effect of soda oil palm EFB lignin on maximal velocity of reaction (V_{max}), Michaelis-Menten constant (K_m) value, and intrinsic clearance (CL_{int}) of 4-MU glucuronidation in RLM was investigated. The K_m, V_{max}, CL_{int}, and K_i values are given in Table 4, while the Lineweaver-Burk plots and secondary plots showing the graphical data for interpretation of inhibition mode are given in Figures 9 and 10, respectively. Based on the analysis, soda oil palm EFB lignin exhibited mixed-type inhibition against 4-MU glucuronidation in RLM, showing the change of apparent V_{max} with only a minor change effect on K_m compared with the control [Table 4]. The K_i value is 7.55 ± 0.78 μg mL^{-1}.

DISCUSSION

In this study, we have investigated the inhibitory effect of three types of oil palm EFB lignin extracts (soda, kraft, and organosolv) and its main oxidation compounds (vanillin, kraft, and organosolv) on *p*-NP and 4-MU glucuronidation. Three different extracts of oil palm EFB lignin were investigated because all of these extracts have their own potential in pharmaceutical and food industry. Our data showed that all oil palm EFB extracts exhibited differential inhibitory effect on *p*-NP glucuronidation either in RLM or RKM. However, oil palm EFB lignin showed a more potent inhibition of UGT activity in RLM compared with RKM. In both RLM and RKM, the inhibitory potency for the extracts was found to be listed in the rank order of soda > kraft > organosolv. Previous study by Mohd Salleh *et al.*[40] reported that the flavonoid content in oil palm EFB lignin was found to be listed in the rank order of soda > kraft > organosolv. Therefore, the data showed that the inhibitory pattern of *p*-NP UGT activity in both RLM and RKM correlated well with total flavonoid content.

For 4-MU glucuronidation study, oil palm EFB lignin also showed a more potent inhibition of UGT activity in RLM compared with RKM. The *in vitro* study using 4-MU as a probe substrate for UGT activity showed that the inhibitory potency for 4-MU glucuronidation in both RLM and RKM can be listed in the rank order of soda > kraft > organosolv. The effect of oil palm EFB lignin on 4-MU glucuronidation in both RLM and RKM also correlated well with flavonoids content of oil palm EFB lignin. Therefore, both studies (the effect of oil palm EFB lignin on *p*-NP and 4-MU glucuronidation) suggest that flavonoids might be responsible for the observed inhibition. The ability of flavonoids as the main constituent

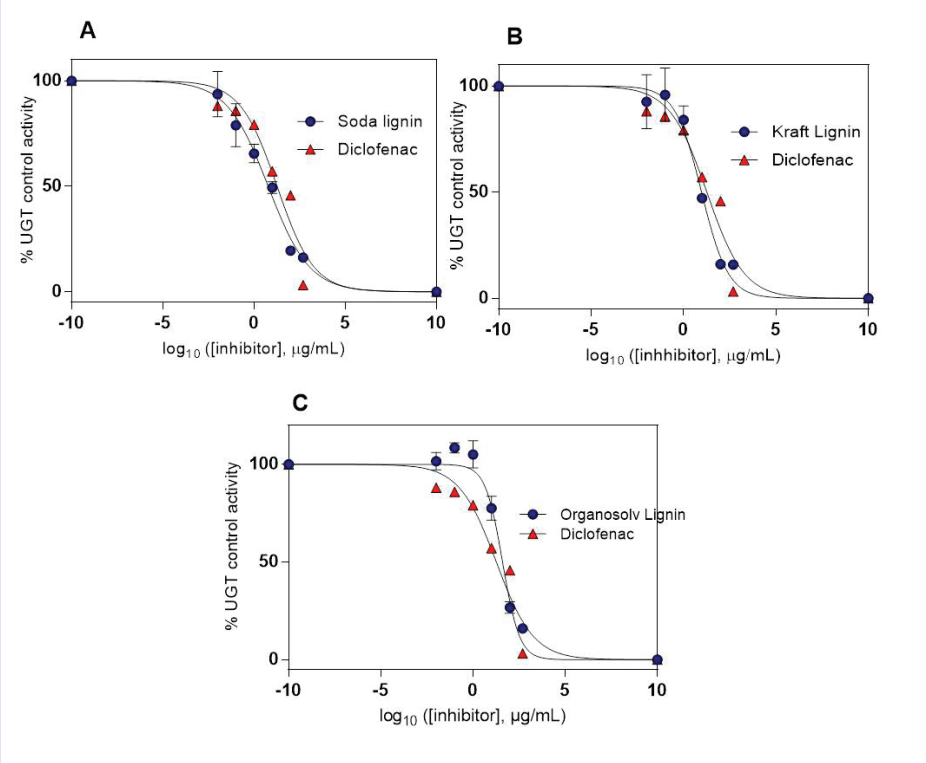

Figure 5: Inhibition of 4-MU glucuronidation in RLM by (a) soda, (b) kraft, and (c) organosolv oil palm EFB lignin compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for three replicate incubations ($n = 3$). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

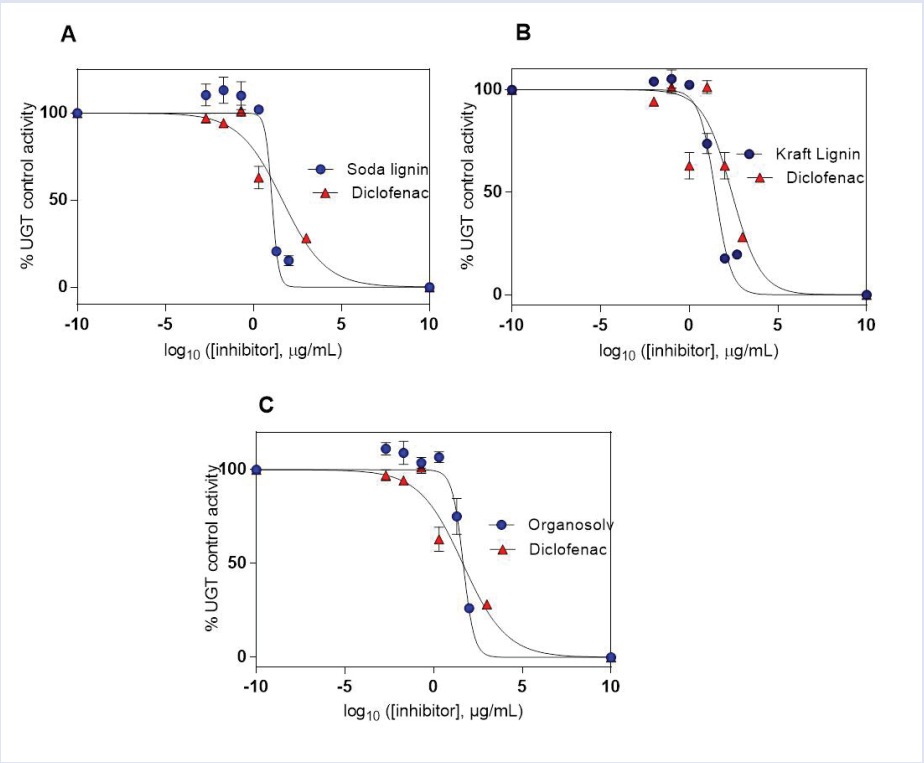

Figure 6: Inhibition of 4-MU glucuronidation in RKM by (a) soda, (b) kraft, and (c) organosolv compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for three replicate incubations ($n = 3$). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

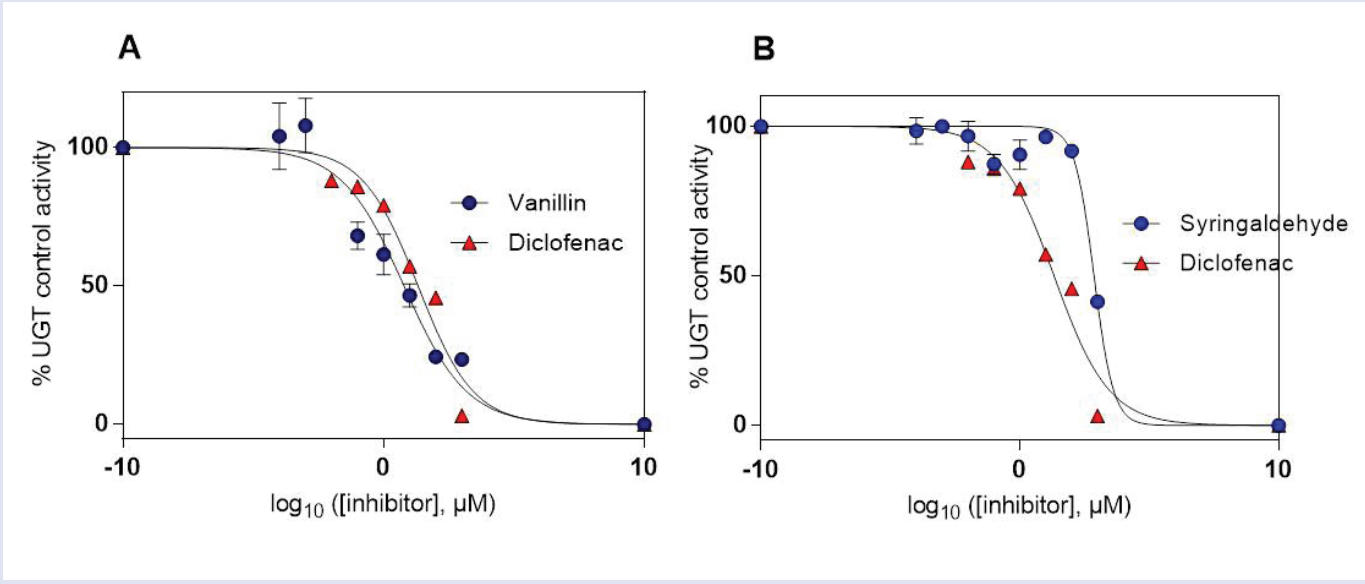

Figure 7: Inhibition of 4-MU glucuronidation in rat liver microsomes by (a) vanillin and (b) syringaldehyde compared with positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for three replicate incubations ($n = 3$). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

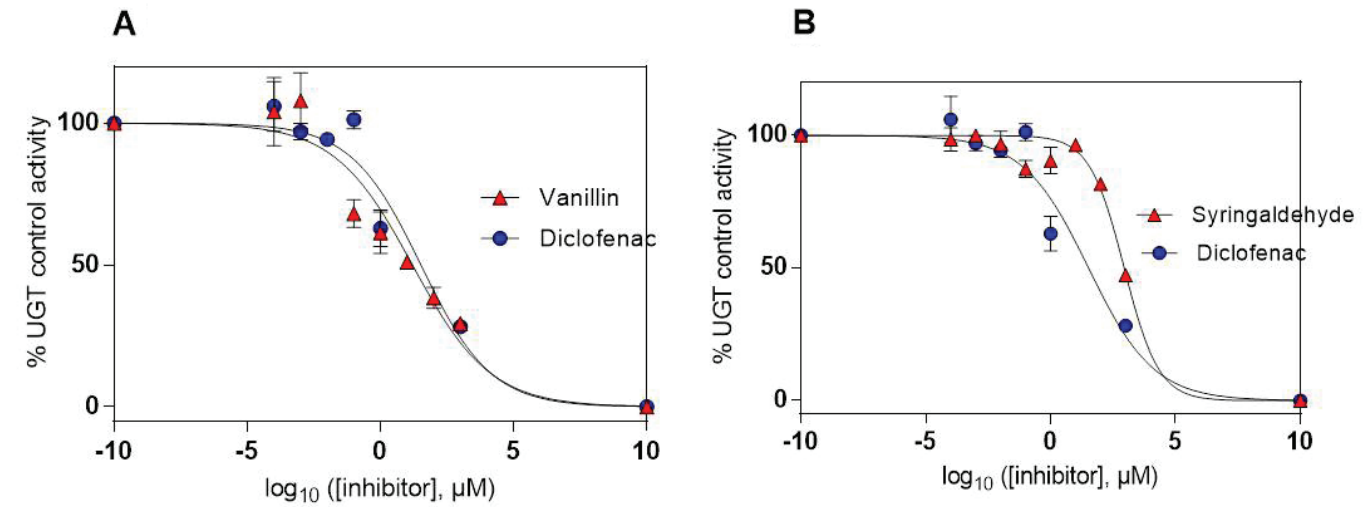

Figure 8: Inhibition of 4-MU glucuronidation in RKM by (a) vanillin and (b) syringaldehyde compared to positive inhibitor (diclofenac). Each data point represents the means of UGT relative activity ± SD for three replicate incubations ($n = 3$). Error bars represent two-sided SD. The goodness-of-fit R^2 value was 0.9 or higher

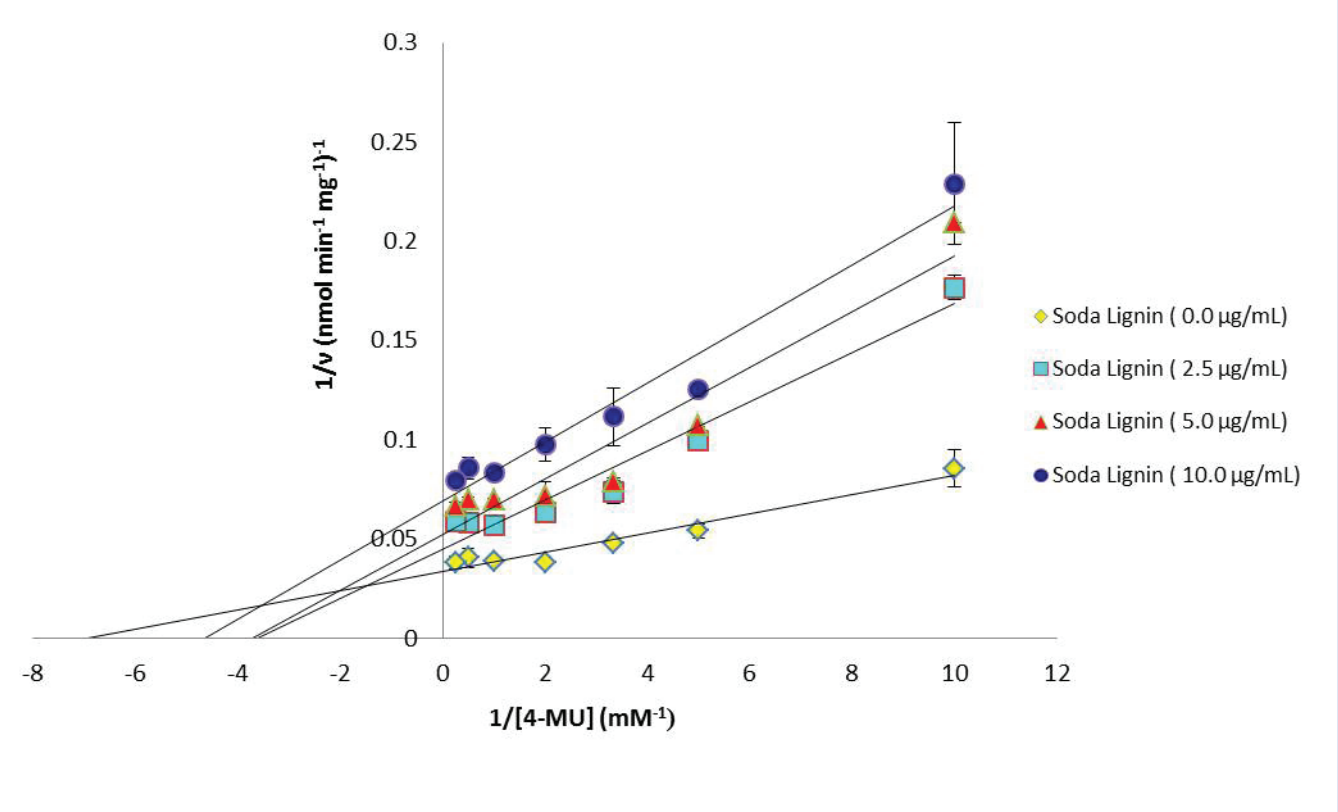

Figure 9: Lineweaver-Burk plots of inhibition of UGT-catalyzed 4-MU glucuronidation by soda oil palm EFB lignin. Each data point represents an average of triplicate measurements (*n* = 3)

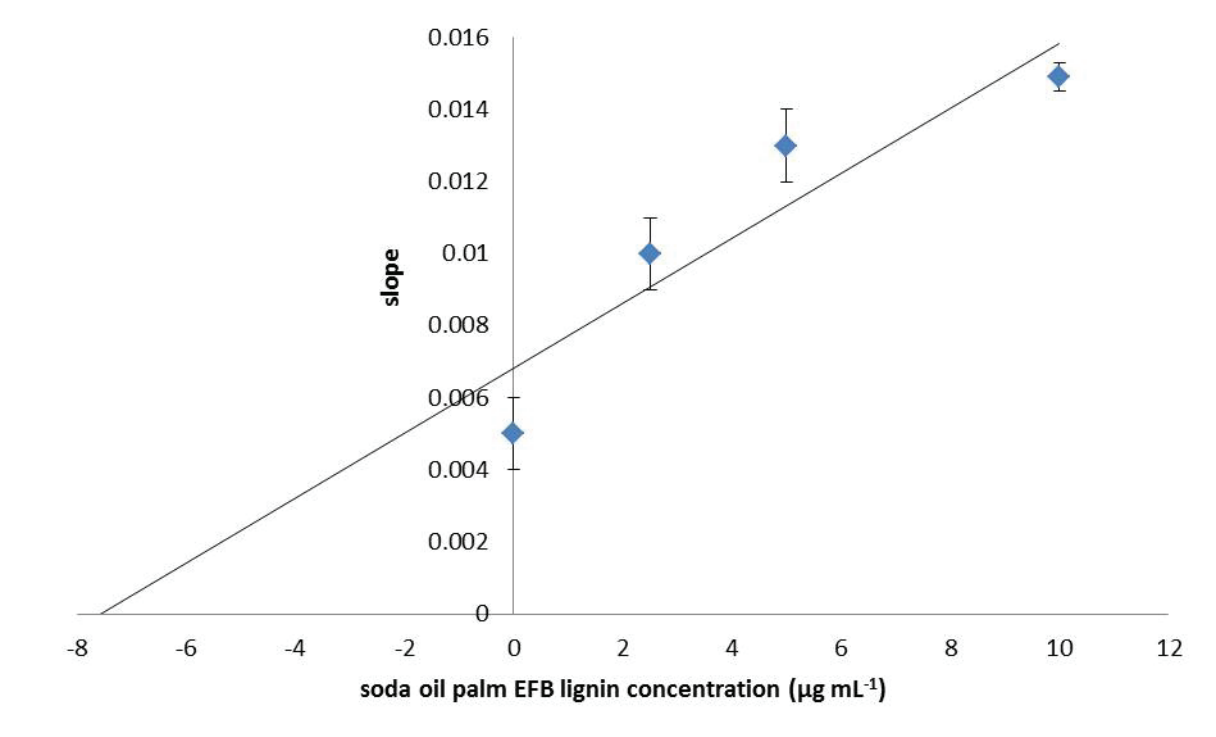

Figure 10: Secondary plots of UGT activity using the slopes of the primary Lineweaver-Burk plots vs. the concentrations of soda oil palm EFB lignin. Each data point represents an average of triplicate measurements (*n* = 3)

that could modulate UGT activity *in vitro* has been reviewed by Moon *et al*.[41] Flavonoids are present in many dietary supplements that were derived from natural plants including *Ginkgo biloba*, soy isoflavones, tea, red wine, and lignin.[40] The effects of flavonoids on enzyme activity are generally dependent on the concentrations of flavonoids present and also different flavonoids ingested.[41] Other research studies on the effect of natural plants such as *Gingko biloba*[42] and *Mitragyna speciosa*[35] on UGT enzymes activity also indicated that flavonoids content was responsible for the inhibition effect.

Incubations with RLM exhibited 1.1-1.5-fold more potent inhibition of *p*-NP glucuronide formation than with RKM by oil palm EFB lignin. However, incubation with RLM exhibited 2.7-4.0-fold more potent inhibition of *p*-NP glucuronide formation than in RKM by oil palm EFB lignin main oxidation compounds [Table 2]. This difference in inhibition potency of liver and kidney suggests that both of them have differentially expressed UGT enzymes in liver and kidney,[33,34] thus contributing to different glucuronidation capacities between liver and kidneys. Incubations with RLM exhibited 1.2-3.1-fold more potent inhibition of 4-MUG formation than in RKM by oil palm EFB lignin [Table 3]. However, the incubation with RLM exhibited 2.6- and 1.2-fold more potent inhibition of 4-MU glucuronidation than in RKM by vanillin and syringaldehyde, respectively [Table 3]. In both RLM and RKM, *p*-hydroxybenzaldehyde showed no notable effect of inhibition for 4-MU glucuronidation (IC_{50} > 1,000 μM).

This difference in inhibition potency of liver and kidney microsomes supported the fact that the expression levels of UGT enzymes in liver microsomes are different compared with kidney microsomes.[45-48] In addition, *p*-NP and 4-MU are planar phenol derivatives. These compounds have been reported to be mainly conjugated by UGT1A6 isoforms in human and rat,[36] although some studies have reported that they are also metabolized by multiple recombinant UGT isoforms.[39,49] Further, it has been suggested that the higher specific activity in RLM compared with RKM is due to the fact that the enzyme activity of UGT1A6 is higher in liver than in kidneys.[50-53]

The inhibitory effect of three main oxidation compounds of oil palm EFB lignin (vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde) on *p*-NP and 4-MU glucuronidation in both RLM and RKM was also investigated in order to examine the relative role, if any, of each compound in the inhibitory effect observed in different oil palm EFB lignin extracts. Our previous study[40] showed that syringaldehyde was the predominant compound in all three types of oil palm EFB lignin, followed by vanillin and *p*-hydroxybenzaldehyde [Table 1]. Meanwhile, as can be seen in Tables 2 and 3, based on the IC_{50} values, vanillin highly inhibits UGT activity of both *p*-NP and 4-MU, whereas syringaldehyde has only weak inhibitory effect and *p*-hydroxybenzaldehyde did not show any notable effect on the activity. Therefore, although syringaldehyde content was the highest percentage in all extracts,[40] it can be concluded that syringaldehyde did not exert a major influence on the inhibitory potency of oil palm EFB lignin on both *p*-NP and 4-MU glucuronidation. Specifically with 4-MU UGT inhibition in RLM, the IC_{50} values of syringaldehyde is 120-fold higher in comparison with vanillin.

Results also suggested that the effect of oil palm EFB lignin on *p*-NP and 4-MU glucuronidation activity in both RLM and RKM was enhanced by the presence of vanillin in all three types of oil palm EFB lignin, as well as the total flavonoid content. Vanillin, which is a food-flavoring agent utilized widely in foods, beverages, drugs, and perfumes, was also found as one of the main oxidation compounds in oil palm EFB lignin.[40-54] Previous study has investigated the UGT conjugation pathway of vanillin by liver microsomes from human, dog, rat, mice, minipig, and cynomolgus monkey.[55] The study showed that vanillin was efficiently glucuronidated by liver microsomes obtained from various species. However, the inhibitory effect of vanillin on UGT DMEs is not documented to date. In summary, this study suggested that the inhibitory effect of oil palm EFB lignin on *p*-NP and 4-MU glucuronidation can be highly attributed to the presence of vanillin in all three types of oil palm EFB lignin.

Further, the effect of 4-MU glucuronidation by soda oil palm EFB lignin in RLM on K_m, V_{max}, CL_{int}, and K_i values and inhibition mode was investigated because it is the most potent inhibitor among all oil palm EFB lignin compounds investigated. Moreover, the HPLC method for the determination of 4-MUG in the 4-MU glucuronidation assay is more specific and sensitive compared with spectrophotometric method that was used on *p*-NP glucuronidation. The Michaelis-Menten constant (K_m) showed the lowest value in 4-MU glucuronidation in RLM control compared with 4-MU glucuronidation in the presence of soda oil palm EFB lignin. The kinetic analysis results showed that 4-MU glucuronidation in RLM without soda oil palm EFB lignin had the highest affinity compared with others. Therefore, it is suggested that the 4-MU glucuronidation activity in RLM may be decreased in the presence of soda oil palm EFB lignin, by an increase in its K_m value, and a decrease in V_{max} value.

The determination of intrinsic clearance (CL_{int}) for drug candidates in the early discovery stage is a common practice in the pharmaceutical industry.[56-58] The intrinsic clearance can be defined as the ability of the liver to remove drug in the absence of flow limitations and binding to cells or proteins in the blood. The *in vitro* CL_{int} may be derived from enzymes' kinetic data such as V_{max}/K_m.[56-61] From the result in Table 4, the CL_{int} value is decreased as the concentration of soda oil palm EFB lignin in 4-MU glucuronidation is increased. The lowest CL_{int} value for 4-MU glucuronidation in RLM with highest concentration of soda oil palm EFB lignin suggests that drugs that undergo the 4-MU glucuronidation may be cleared less efficiently by liver.[62] Therefore, soda oil palm EFB lignin exhibited strong inhibition on UGT activity in RLM with mixed-type inhibition mode.

The mechanism of mixed-type inhibition by the plant extracts with regards to UGT enzymes is also shown by many herbs and plant extracts.[63,64] Mohamed and Frye[63] reported that the incubation of ginkgo flavonoids, that is, quercetin (in human liver and intestine microsome) and kaempferol (in human intestine microsome) exhibited mixed type of inhibition toward mycophenolic acid (MPA) glucuronidation. Zainal Abidin *et al*.[64] reported that the incubation of *Andrographis paniculata* extracts and its active constituent (neoandrographolide) exhibited mixed mode of inhibition toward *p*-NP glucuronidation in UGT1A1.

CONCLUSION

The effect of oil palm EFB lignin and its main oxidation compounds on *p*-NP and 4-MU glucuronidation were successfully investigated in this study. Based on the data, the inhibitory potential of the investigated extracts for *p*-NP and 4-MU glucuronidation in RLM and RKM can be listed in the following rank order: soda > kraft > organosolv. For compounds, the rank order is vanillin > syringaldehyde > *p*-hydroxybenzaldehyde. Results suggested that the effect of oil palm EFB lignin on *p*-NP and 4-MU glucuronidation activity in both RLM and RKM was enhanced by the presence of vanillin as well as the total flavonoid content. Based on our findings, oil palm EFB lignin may inhibit glucuronidation of substrate by UGT enzymes especially UGT1A6 particularly in rat liver. These findings suggest interactions of oil palm EFB lignin with UGT1A6 substrates are possible. However, future clinical studies are warranted to evaluate the *in vivo* pharmacokinetic relevance of these interactions.

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Conflicts of interest

There are no conflicts of interest.

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