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Chapter 12

PREPARATION OF DEHYDROGENATION POLYMER FROM ISOEUGENOL AND BIOLOGICAL ACTIVITY CHARACTERIZATION

Yimin Xie[1,2,*], Xuekuan Chen[1], Houkuan Zhao[1], Chen Jiang[1], Hongfei Wu[1], Shuying Bi[1] and Zhezi Ye[1]
[1]Research Institute of Pulp and Paper Engineering,
Hubei University of Technology, Wuhan, China
[2]Hubei Provincial Key Laboratory for Green Light Industry,
Hubei University of Technology, Wuhan, China

ABSTRACT

To understand the relationship between the chemical structural characteristics of dehydrogenation polymer (DHP) and its antibacterial and anticancer activities, DHP with low molecular weight was prepared *in vitro* with isoeugenol (IEG) as a precursor of lignin biosynthesis using laccases as the catalyst by a bulk method. The chemical structure of DHP was analyzed with Fourier-transform infrared spectroscopy (FTIR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy. The antibacterial and anticancer activities of the four DHP fractions with low molecular weight extracted by petroleum ether, diethyl ether, ethanol and acetone were also investigated. The FTIR spectra suggested that the isoeugenol could be polymerized to guaiacyl-type DHP. Analysis of the ^{13}C-NMR spectra indicated that the DHP was mainly composed of β-5, β-O-4, β-1, β-β and 5-5 subunits. The results of the determination of antibacterial activity showed that the four DHP fractions had a high content of phenolic hydroxyl group, showed strong antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. The cytotoxicity assay demonstrated that the low molecular weight fraction extracted by ether had obvious anticancer activity. These results showed that DHP with low degree of polymerization had good biological activity.

* Corresponding Author Email: ppymxie@163.com.

Keywords: dehydrogenation polymer, isoeugenol, ^{13}C-NMR, antimicrobial activity, anticancer activity

INTRODUCTION

Lignin, one of the three main components that occurs naturally in higher plants, is a high-molecular weight polymer of phenolic compounds with a complex structure [1]. Deposited in the cell wall, lignin is necessary for both structural support as well as preventing microbial attack [2]. Lignin is polymerized by three monolignols, i.e., *p*-coumaryl, coniferyl and sinapyl alcohols, with lignin oxidase as the catalyst [3, 4]. As one of the possible phenolic monomers of native wood lignin, isoeugenol is a compound that has a guaiacyl structure [5]. To investigate the occurrence of lignin, dehydrogenation polymer (DHP) synthesized with isoeugenol has been used as a lignin model to explore the lignin structures [6-9]. The biosynthesis of isoeugenol has been elucidated recently in some plants where isoeugenol is produced at a high level [10, 11]. It has been shown that isoeugenol can be synthesized from the monolignol of coniferyl alcohol by a specific enzyme, which results in acylation of side-chains [12]. It has been proposed that isoeugenol biosynthesis is involved in the lignin biosynthetic pathway.

The structure of DHP synthesized from isoeugenol has been studied only rarely. Salanti [9] analyzed the lignin-like polymer resulting from isoeugenol radical coupling and found that the characteristic signals of intermonomeric bonds correlated to the β-carbon and α-carbon of β-0-4 and β-5 moieties, respectively, by carbon-13 nuclear magnetic resonance (^{13}C-NMR). Ye [13] studied the polymerization of isoeugenol in the presence of polysaccharides and found that the generated DHP contained β-1, β-β, β-5 and β-O-4 structures. Hunay [14] performed mild oxidative degradation of the isoeugenol polymer and Björkman's milled wood lignin (MWL) and he found that guaiacyl compounds were produced in both of the samples. Combined with further experimental results, it was confirmed that the isoeugenol polymer was revealed to be of striking similarities to natural lignin [15].

Plant polyphenols are natural products and have high antioxidant, antibacterial and other biological activities [16]. Lignin as a type of plant polyphenolic compound contains a large number of chemical functional groups, such as phenolic hydroxyl, aliphatical hydroxyl, carboxyl, carbonyl and methoxy group. It has been suggested that phenolic hydroxyl and methoxy groups have certain biological activities [17]. Pan [18] analyzed 21 kinds of solvent lignin from the same hybrid poplar and found that the ethanol soluble fractions had the strongest antioxidant properties. Compared to other fractions, these fractions contained more phenolic hydroxyl groups and less alcoholic hydroxyl groups with lower molecular weight and narrower polydispersity. A similar result was found in the studies by Hage [19] and Ma [20]. They obtained the low molecular weight fraction of

MWL in the solvent phase by organic solvent extraction of wood meal, while the fraction with higher molecular weight was rich in precipitate [21]. Compared to a series of guaiacyl- and syringyl-type lignin and lignin derivatives, isoeugenol was also proved as notable antimicrobial [22, 23]. It has been proposed that the bacteriostatic actions of isoeugenol operate against a variety of bacteria, such as *Escherichia coli*, *Bacillus licheniformis* and *Staphylococcus aureus* [22, 24]. Paper packaging has been endowed with antimicrobial properties by modification with DHPs obtained from isoeugenol [25]. Espinozaacosta [26] summarized the research reports concerning the biological activities of technical lignin and found that lignin exerts potential antimutagenic properties. However, there are few detailed studies on the biological activity of the DHP fractions with different molecule weights obtained from isoeugenol.

In the present study, isoeugenol was polymerized to DHP using a bulk method and catalyzed by laccase. The obtained DHP was further fractionated with different organic solvents into fractions with different molecular weight. The chemical structures of the products were characterized with FTIR and ^{13}C-NMR spectroscopy. The antimicrobial and anticancer activities of the obtained DHP were also investigated.

EXPERIMENTAL

Materials

Isoeugenol (98%) was purchased from Sigma Co. Ltd. Laccase (No. 51003, 1000 IU/mL, determined by the methods of Fukushima and Kirk [27]) was obtained from Novazyme Co. Ltd. All other chemicals were of analytical grade.

Gram-negative bacterium, *E. coli* ATCC 25922 and gram-positive bacterium, *S. aureus* CMCC(B) 26003 were obtained from Shanghai Luwei Technology Co. Ltd. and used as test organisms. The bacterial inoculums were prepared to obtain a bacterial suspension in 5 mL of nutrient broth. The common nutritional agar culture medium was purchased from Aobox Biotechnology Company and used for the agar plates. HeLa cells were obtained from Shanghai Xinyu Biotechnology Co. Ltd. and used to detect the antitumour properties of the fractions. An autoclave was used for sterilization and media preparation at 121°C for 20 min.

Methods

Synthesis of DHP

Five grams of isoeugenol was dissolved in 50 mL mixture (1:1, v/v) of ethanol and acetate buffer (0.1 M, pH = 5.0), and mixed with 1 mL laccase (1000 IU/mL). During the

reaction, the mixture was bubbled with sterile air for the required oxygen and maintained in a water bath at 30°C. After 24 h, the crude product was collected by centrifugation and washed with distilled water. After being freeze dried, the crude product was dissolved in 60 mL dichloroethane/ethanol (2:1, v/v) by stirring at 20°C for 6 h in order to remove the contaminant of the enzyme. The supernatant was collected by centrifugation and DHP was obtained after removing the solvent by rotary evaporation *in vacuo*.

Fractionation of the DHP

As shown in Figure 1, the DHP was extracted by petroleum ether, diethyl ether, ethanol and acetone. Four grams of the DHP was suspended in 200 mL of petroleum ether with magnetic stirring. By centrifugation, the petroleum ether solution fraction (F_1) was obtained as the supernatant followed by rotary evaporation with a yield of 25.6%. The above precipitate was further fractionated with diethyl ether, ethanol and acetone using the same method. Fractions F_2, F_3 and F_4 were obtained with yields of 63.9%, 2.5% and 1.0% respectively.

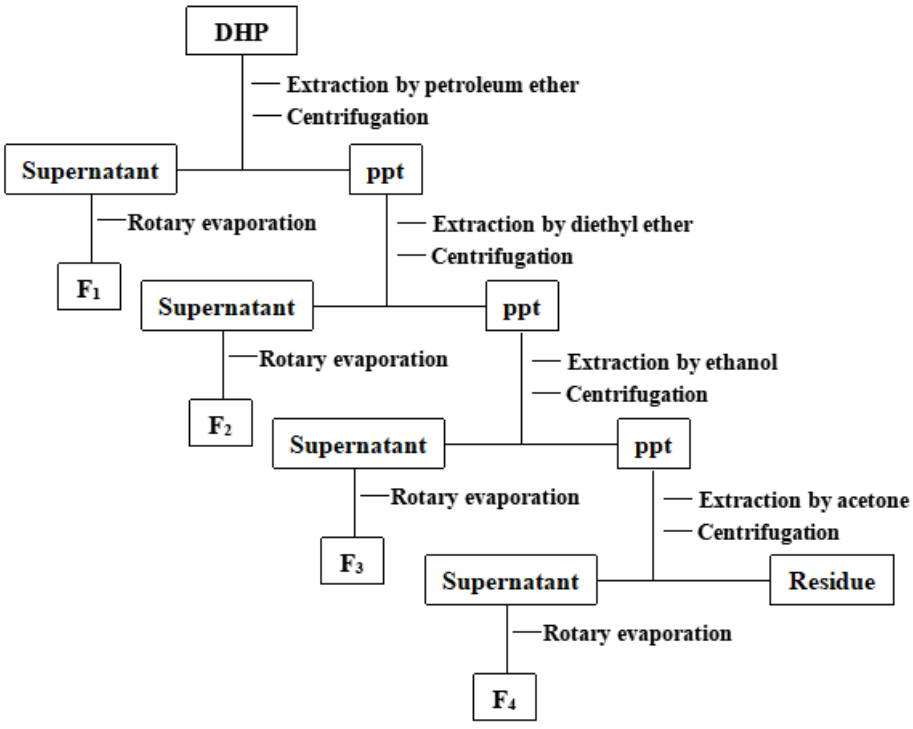


Figure 1. Fractionation procedure of the DHP.

Molecular Weight Determination

Two milligrams of the F_1, F_2, F_3 and F_4 fractions were dissolved in high-performance liquid chromatography grade tetrahydrofuran and filtrated by 0.22 μm membrane filter.

The molecular weights were determined by gel permeation chromatography (GPC) with a column of Shim-pack GPC-803D (Size: 300 mm × 8 mm ID). Tetrahydrofuran was applied as mobile phase with a flow rate of 0.6 mL/min. Column temperature was 30°C and injection volume was 25 μL.

Determination of Total Phenol Content

Based on the method by Christel Quettier-Deleu [28], the total phenol content of each fraction was determined. Seven milliliters of distilled water, 0.5 mL phenolic phenol reagent and 0.5 mL ethanol solution of catechol were mixed and shaken for 3 min. After 2 mL 20% Na_2CO_3 solution was added, the mixture was heated for 1 min in a 100°C water bath and cooled naturally to 20°C. Then, its absorbance was determined with a UV-Vis spectrometer at 685 nm. A standard curve was plotted for the concentration of catechol and absorbance. By repeating the above steps, F_1, F_2, F_3, F_4 and isoeugenol (IEG) were tested to determine the total phenol content.

Analysis of the Structure of the DHP

The FTIR spectrum of the DHP was obtained with a Thermofisher Nicolet 6700 FTIR spectrometer using the KBr pellet technique. The samples were scanned 32 times in the range of 4000 to 500 cm^{-1}.

The liquid ^{13}C-NMR spectrum of the sample was determined at 100.6 MHz on a Varian oneProbe 400 NMR spectrometer. The sample was placed in a φ5 mm determining tube and dissolved in 0.6 mL dimethyl sulfoxide (DMSO)-d_6 solvent. Pulse delay was 1.75 s and acquisition time was 0.9 s. The sample was scanned approximately 20000 times.

Evaluation of Antimicrobial Activity

Antimicrobial activities of the DHP fractions were tested using the filter paper agar diffusion method [29]. Antimicrobial activities were determined by their zone of inhibition. The bacterial suspension was diluted with sterile saline to 1.5×10^8 CFU/mL. Two hundred microliters of bacterial suspension was uniformly coated onto every agar plate. The four DHP fractions were dissolved in DMSO to obtain a series of solutions with concentration of 5 mg/mL to 2.5 mg/mL. Dried sterile filter papers of 6 mm in diameter that had been prepared previously were immersed in the above solutions for 6 h. Then, the filter papers were removed and attached onto the agar plates, followed by the addition of 10 μL of the corresponding sample solutions onto the surface of the filter papers. After culture at 37°C for 16 h, the zone of inhibition was observed and measured.

Evaluation of Anticancer Activity

Anticancer activity of the four DHP fractions were detected by the 3- (4,5- dimethyl-2- thiazolyl) - 2,5-diphenyl-2-H-tetrazolium bromide (MTT) method [30]. HeLa cells were cultured in Roswell Park Memorial Institute 1640 medium, which was supplemented with

100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 37°C and 5% CO_2 in a humidified incubator. HeLa cells (1×10^6 cells/mL) were pre-treated with various concentrations of the four DHP fraction solutions or solution medium (1% ethanol) for 24 h. The cells were rinsed and incubated with MTT (5 mg/mL) for 4 h. The culture solution was discarded and the formazan blue formed in the cells was dissolved with DMSO. The absorbance at 490 nm was measured in a microplate reader. The absorbance of the formazan formed in the intact cell was taken as the controls. Finally, the semi-inhibition concentration ($IC_{50\%}$) was calculated via the curves.

RESULTS AND DISCUSSION

Molecular Weight of the DHP

The molecular weights of the four DHP fractions, i.e., F_1, F_2, F_3 and F_4 are shown in Table 1. The average molecular weights (M_w) of the F_1, F_2, F_3 and F_4 fractions were 330, 621, 1211 and 3670, respectively.

Table 1. Molecular weight of the fractions of the DHP

Fractions	M_w	M_n	M_w/M_n	TPC(mg catechol/g sample)
F_1	330	126	2.6	149
F_2	621	237	2.6	104
F_3	1211	449	2.7	93.5
F_4	3670	1579	2.3	80

Note: TPC: Total phenol content.

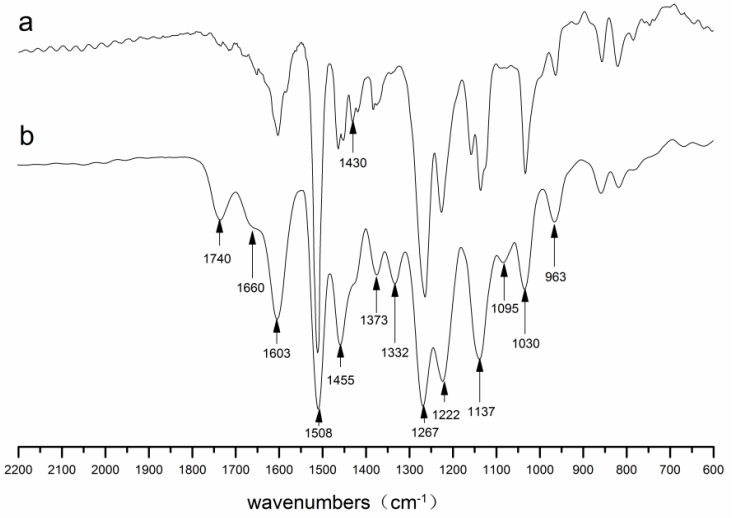


Figure 2. FTIR spectra of isoeugenol (a) and the DHP (b).

The molecular weight of the fractions increased with the solubility of the solvent. Because the molecular weight of monomeric compound IEG was 162, the main components of the four fractions can be calculated to be dimer, tetramer, heptamer and dodecamer. The dispersion coefficient (M_w/M_n) of the four components of F_1, F_2, F_3 and F_4 were approximately 2.6, which means there were a lot of low molecular weight substances in the DHP fractions. The total phenol contents of the F_1, F_2, F_3 and F_4 were 149, 104, 93.5 and 80 mg catechol/g sample, respectively, which indicated that the total phenol content decreased with the increase of molecular weight.

FTIR Analysis of the DHP

As shown in Figure 2, FTIR spectroscopy was used to analyze the DHP from isoeugenol to reveal the chemical structure in the region between 2200 cm^{-1} and 600 cm^{-1}. Based on the assignment reported previously [6, 13, 14], the specific absorption associated with lignin assigned to aromatic skeletal vibrations were located at 1509 cm^{-1} and 1600 cm^{-1}, as shown in Figure 2. Furthermore, the stretching vibration of the guaiacyl rings was at a lower wavenumber range at 1267 cm^{-1}. However, a strong band at 1740 cm^{-1} which was identified as unconjugated carboxyl stretching only appeared in the DHP spectrum. This indicated the possibility of the formation of carboxyl groups by oxidation of isoeugenol during polymerization catalyzed by laccase. Moreover, the peak at 963 cm^{-1} was characteristic of a conjugated double bond in dehydrodiisoeugenol, which could not be found in the spectrum of MWL [6].

Analysis of the DHP by ^{13}C-NMR Spectroscopy

The ^{13}C-NMR spectrum of DHP was shown in Figure 3. The assignment of signal absorption peaks was shown in Table 2, and the structural units of DHP were shown in Figure 4. The signal at 172.2 ppm (No. 2) could be assigned to γ-COOH (**I**, Figure 4) of cinnamic acid. The peak at 169.8 ppm (No. 3) was C_α = O (**IV** and **V**, Figure 4) of vanillic acid and its derivative. These two different carbonyl groups indicated that DHP had a different degree of oxidation in the side chain during the polymerization process [8]. The signal at 152.4 ppm (No. 4) was from C_3 in etherified 5-5 structure (**X**, Figure 4). In addition, signals at 130 to 134 ppm (No. 9-11) were mainly from C1 in β-O-4, β-5 and 5-5. At 128.4 ppm (No. 12) there was a weak signal, which came from C_α and C_β in -C_α = C_β- structure in the coniferyl alcohol (**II**, Figure 4) and showed that a small amount of γ-CH_3 of isoeugenol was oxidised to γ-CH_2OH. From 110 ppm to 125 ppm, there was a large amount of signal, which was mainly from C_5 and C_6 of the aromatic ring of lignin [31, 32].

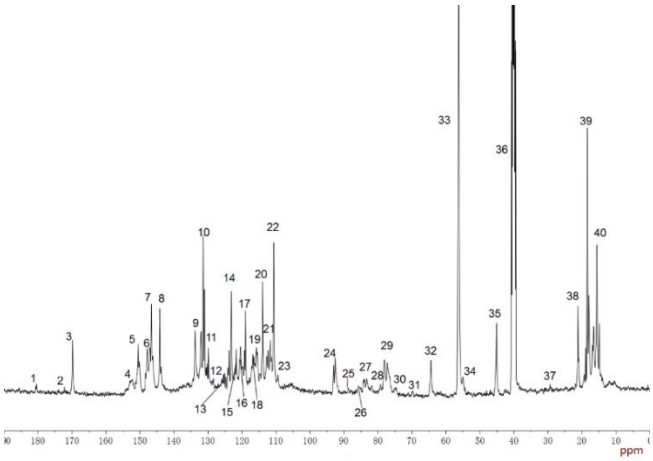
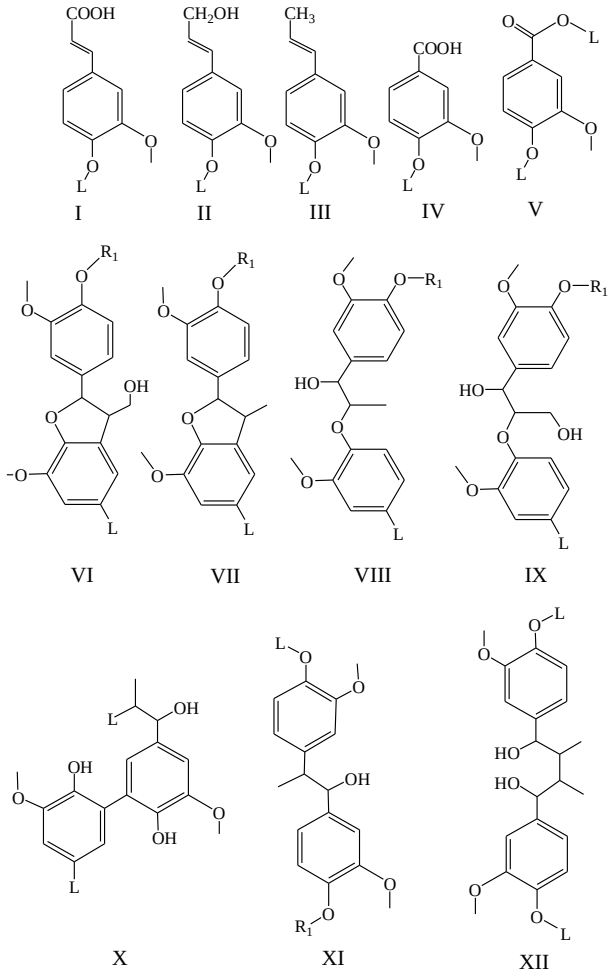


Figure 3. ^{13}C-NMR spectrum of the DHP.



Note: R_1=H or alkyl; L=polylignol

Figure 4. Substructures of the DHP.

The obvious signal at 93.0 ppm (No. 24) was the resonance of Cα in β-5 with -$C_\gamma H_3$ (**VII**, Figure 4). This demonstrated that the DHP contained large amount of β-5 structure. The signal at 88.7 ppm (No. 25) was the resonance of C_β in β-1 (**XI**, Figure 4), which indicated that the DHP contained only small amount of β-1. It was suggested that the DHP contained β-β (**XII**, Figure 4) as C_β in β-β appeared at 85.7 ppm (No. 26). The signal at 78.1 ppm (No. 29) was C_α in β-O-4, which demonstrated that DHP contained a large amount of β-O-4 with γ-CH_3 (**XIII**, Figure 4). This meant that a lot of β-O-4 could be synthesized by dehydrogenation polymerization of isoeugenol even with the bulk method [9, 15]. Signal No. 30 can be assign to C_α in β-O-4 with γ-CH_2OH (**IX**, Figure 4). A weak signal at 55.0ppm (No. 34) was from C_β of β-5 with γ-CH_2OH (**VI**, Figure 4). In addition, there was a strong signal from Cγ of β-5 with -$C_\gamma H_3$ at 45.1 ppm (No. 35). This proved that the content of β-5 in DHP was high. The strong signals at 18.4 ppm (No. 39) and 15.6 ppm (No. 40) could be assigned to the γ-methyl groups of β-5 and β-O-4 in the DHP. From the spectral analysis, it could be concluded that the main structural units in the DHP were β-5, β-O-4, β-1, β-β and 5-5. These were very similar to the structural units of protolignin.

Investigation of Antibacterial Activity

The DHP obtained from isoeugenol was hydrophobic, *i.e.*, could only be dissolved in organic solvents such as DMSO. Therefore, the antibacterial activity of DMSO was first determined. It was found that DMSO could not inhibit the growth of *E. coli* and *S. aureus*. Therefore, DMSO was suitable to dissolve the DHP samples in the antibacterial experiment.

The zones of inhibition of bacteria were shown in Figure 5. The antibacterial activities of the four DHP fractions (F_1 ~ F_4) were presented in Table 3 and Table 4. The F_1 and F_2 fractions with concentration of 5 mg/mL and 2.5 mg/mL, respectively, inhibited the growth of *E. coli* and *S. aureus*, resulting in an obvious zone of inhibition. However, the experiments with the F_3 and F_4 fractions did not show clear inhibition zone. Table 4 showed that the F_1 and F_2 fractions produced zones of inhibition on gram-positive *S. aureus* (8 mm and 9 mm, respectively), whereas zones of inhibition of 7–8 mm appeared on gram-negative *E. coli* as shown in Table 3.

These results indicated that the antibacterial compounds of the DHP were mainly low molecular weight fractions that were soluble in petroleum ether and diethyl ether. The structures of the side chain containing a double bond at the α and β positions, and/or a methyl group at γ might play a considerable role in antimicrobial activity [22]. Most of these structures might remained in the F_1 and F_2 fractions. The antibacterial experiments proved that the DHP fractions with low molecular weight had strong antibacterial activity

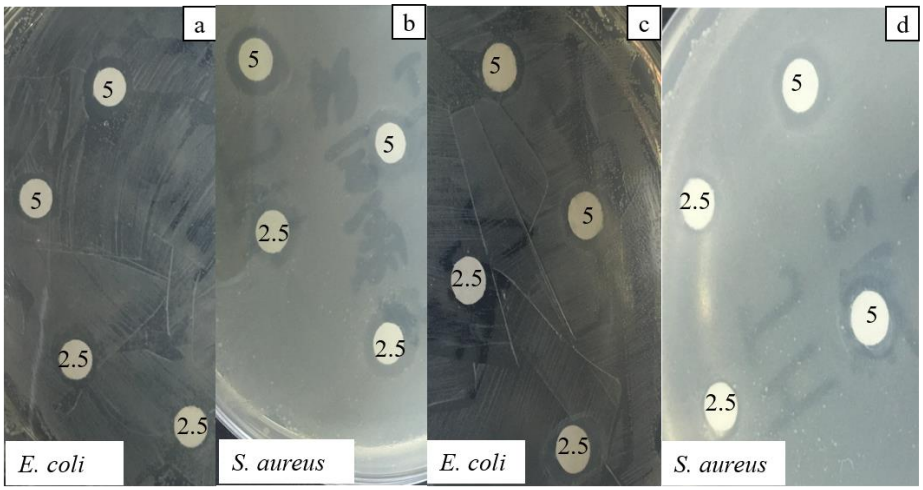
against *E. coli* and *S. aureus*. Moreover, it provided a theoretical basis for lignin modification and application.

Table 2. Assignments of ^{13}C NMR spectrum of the DHP

Signal	δ (ppm)	Assignments
1	180.4	O-C=O in carboxylic acid
2	172.2	C*α* in cinnamic acid
3	169.8	Cα=O in benzoic acid
4	152.4	C_3 (etherified 5-5)
5	150.5	C_3(β-O-4)
6	147.8	C_3(β-5)
7	146.7	C_4(β-5) or C_3(β-O-4)
8	144.1	C_4(5-5)
9	133.8	C_1(β-O-4)
10	131.4	C_1(β-5)
11	129.9	C_1(5-5)
12	128.4	$C_α$ and $C_β$ (coniferyl alcohol)
13	125.1	C_5(5-5)
14	123.1	C_6(β-β)
15	121.7	C_6(5-5)
16	120.4	C_1(β-1) or C_1(etherified β-O-4)
17	119.0	C_6(β-5) or C_6(β-O-4)
18	116.8	C_5(β-β)
19	115.8	C_4(β-β)
20	113.9	C_5(β-O-4)
21	111.7	C_2(β-O-4)
22	110.6	C_2(β-5)
23	109.4	C_2(5-5)
24	93.0	$C_α$(β-5)
25	88.7	$C_β$(β-1)
26	85.7	$C_β$ (β-β with -$C_γCH_3$)
27	84.0	Cα(etherified β-O-4)
28	79.2	$C_β$(etherified β-O-4) or Cα(β-β)
29	78.1	$C_β$(β-O-4 with -$C_γCH_3$)
30	74.8	$C_α$(β-O-4 with $C_γ$-CH_2OH)
31	69.8	unknown
32	64.4	$C_γ$(β-5 with $C_γ$-CH_2OH)
33	56.5	-OCH_3
34	55.0	$C_β$(β-5 with $C_γ$-CH_2OH)
35	45.1	$C_γ$ (β-5 with -$C_γCH_3$)
36	40.5	DMSO
37	29.4	-CH_3
38	21.2	-CH_3
39	18.4	$C_γ$(β-5 with -$C_γH_3$)
40	15.6	$C_γ$(β-O-4 with -$C_γH_3$)

Table 3. Activities of the DHP fractions against *E. coli*

Concentration gradient(mg/ml)	Zone of inhibition(mm)			
	F_1	**F_2**	**F_3**	**F_4**
2.5	8	7	--	--
5	8	7	--	--



Legend: F_1: Figure 5a and Figure 5b; F_2: Figure 5c and Figure 5d. The 5 and 2.5 represent concentration of solution in mg/mL.

Figure 5. The inhibition zone of the F_1 and F_2 fractions of the DHP.

Table 4. Activities of the DHP fractions against *S. aureus*

Concentration gradient(mg/ml)	Zone of inhibition(mm)			
	F_1	**F_2**	**F_3**	**F_4**
2.5	9	8	--	--
5	9	8	--	--

Investigation of Anticancer Activity of the DHP Fractions

As shown in Figure 6 and Table 5, the four DHP fractions (F_1 ~ F_4) had an inhibitory effect on the proliferation of HeLa cancer cells. With an increase in concentration, the effect of growth inhibition was more obvious. The lower molecular weight fractions extracted by petroleum (F_1) and diethyl ether (F_2) showed excellent anticancer activity, with semi-inhibitory concentrations ($IC_{50\%}$) of 59.764 μg/mL and 16.146 μg/mL, respectively. The inhibition effect of high molecular weight fractions was not significant. The anticancer activities were relevant to the phenolic hydroxyl structure in DHP fractions with low molecular weight. Lignin is one of the polyphenolic compounds in plants and

phenolic hydroxyl has a strong anti-free radical capacity. The oligomers with low molecular weight from DHP polymerized by isoeugenol led to a high content of free phenolic hydroxyl groups [26, 33].

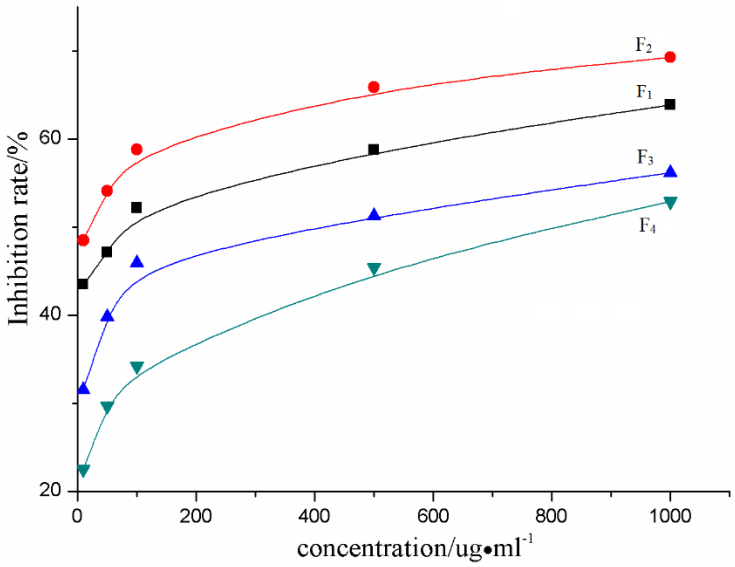


Figure 6. Relationship between concentrations of the DHP fractions and inhibition rates.

Table 5. $IC_{50\%}$ values of the four DHP fractions

Fractions of DHP	**F_1**	**F_2**	**F_3**	**F_4**
$IC_{50\%}$ (μg/ml)	59.764	16.146	317.697	822.731

CONCLUSION

1. The FTIR analysis showed that the isoeugenol precursor was polymerized to be DHP. By analyzing the ^{13}C-NMR spectrum of the DHP, it was found that the phenylpropane units of the DHP were mainly connected by β-O-4, β-β, β-5 and β-1 linkages with the existence of minor subunits as ferulic acid, isoeugenol and coniferyl alcohol. These results proved that the association between phenylpropane units in the DHP from isoeugenol was similar to that of protolignin.
2. The antibacterial experiments showed that the DHP fractions with low molecular weight had strong antibacterial activities against *Escherichia coli* and *Staphylococcus aureus*. This result provided a theoretical basis for lignin modification and application.
3. The four DHP fractions classified by petroleum ether, diethyl ether, ethanol and acetone obviously inhibited the proliferation of Hela cancer cells, especially for

the diethyl ether extracted fraction F_2. This effect may be related to the high content of free phenolic hydroxyl in its structure.

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