Preparation of Oligomeric Dehydrogenation Polymer and Characterization of its Antibacterial Properties

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To investigate the relationship between the chemical structural characteristics of lignin and its antibacterial activity, a low molecular weight dehydrogenation polymer (DHP) was synthesized in vitro with isoeugenol as a precursor and catalyzed by laccase. The DHP was fractionated to obtain a petroleum ether-soluble fraction (F1), diethyl ether-soluble fraction (F2), ethanol-soluble fraction (F3), and acetone-soluble fraction (F4). The results of antibacterial experiments showed that only F1 and F2 could effectively inhibit the growth of Escherichia coli and Staphylococcus aureus. Furthermore, nine compounds (Z1 to Z9) were obtained via the column chromatographic separation from F1 and F2. Mass spectrum analysis results showed that all of these compounds contained a β-5 structure. Antibacterial experiments showed that dimers (Z1 and Z2) could inhibit both S. aureus and E. coli. The trimers, tetramers, and pentamers (Z3 to Z9) could inhibit S. aureus but had no inhibitory effect on E. coli. The aldehyde groups and the condensed 5-5 structure decreased the antibacterial properties of DHP, whereas the presence of the β-5 structure may be related to the antimicrobial ability of DHP.

Keywords: Lignin; Oligomer; Dehydrogenation polymer; Antibacterial activity; Structure

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INTRODUCTION

Lignin, one of the three major cell wall components of plants, is a major source of natural antibacterial substances (Hatakeyama and Hatakeyama 2009; Gyawali and Ibrahim 2014). It was defined as a component comprising of 11 different phenolic fragments that inhibited the growth of microorganisms such as Escherichia coli, yeast, and Aspergillus (Jung and Fahey 1983; Zemek et al. 1979). Previous research has indicated that the antibacterial properties of fibrous plants, such as hemp, are mainly derived from their cannabinoids, alkaloids, and phenolic compounds. Recently, Afrin et al. (2012) established that the antibacterial compound of bamboo was lignin, rather than hemicellulose or other water-soluble compounds. Furthermore, the antibacterial properties of lignin have been related to its phenolic components. Unbleached kraft pulp could react with different phenolic compounds (isoeugenol, butyl p-hydroxybenzoate, p-coumaric acid, and ferulic acid) in the presence of laccase to provide new antibacterial properties (Pei et al. 2012). The obtained phenolic substances accounted for the antibacterial activity because they were able to destroy the bacterial membrane or change its permeability (Barber et al. 2000). However, the structure and biological activity of natural lignin was shown to be greatly influenced by the separation and extraction methods used, and the molecular weight of the obtained product. Pan et al. (2006) studied
the antioxidant capacity of 21 organosolv ethanol lignin samples; they found that processing conditions affected the functional groups and molecular weight of the extracted organosolv ethanol lignins and consequently influenced the antioxidant activity of the lignins. Sláviková and Košíková (1994) compared the inhibitory effect of lignin obtained from different sources and the inhibitory effect of treated lignin on yeast. The results showed that lignin from different sources had different bacteriostatic properties, while the inhibitory effect of treated lignin on yeast was stronger than that of untreated lignin.

Researchers have synthesized lignin by simulating a plant-like environment (Ropponen et al. 2011; Arshanitsa et al. 2013; Boeriu et al. 2014). The method of obtaining lignin by enzyme-catalyzed polymerization of lignin precursor has been gradually adopted. Yang and Xie (2008) used coniferin as a raw material to synthesize lignin dehydrogenation polymer (DHP) by both the bulk and dropwise methods. They found that the content of β-5 in the DHP obtained via the bulk method was slightly higher, while the content of β-O-4 in the DHP obtained via the dropwise method was higher. Furthermore, the relative molecular mass of the DHP obtained by the bulk method was lower. Isoeugenol (IEG) is a natural product that is part of clove oil and cinnamon oil. It has been industrially produced by isomerization of eugenol. Compared with the lignin basic monomer of coniferyl alcohol, isoeugenol has similar phenylpropane structure. The only difference is that the substituent at the γ-position of the side chain is methyl. In recent years, the biosynthesis of isoeugenol has been confirmed in fairy fans (Clarkia breweri) and petunia (Petunia × hybrida). Isoeugenol was synthesized by side chain acetylated coniferyl alcohol under enzyme action (Koeduka et al. 2006; Muhlemann et al. 2014). In addition, as early as 1989, isoeugenol was used as a lignin precursor to study lignin in many studies, and the synthetic DHP was similar to natural lignin (Hunay 1989). Ye et al. (2016) synthesized low molecular weight DHP with isoeugenol as a precursor and confirmed that β-O-4, β-β, β-5, and β-1 were the main structures of DHP. However, most of their work remained at the chemical structural stage. There have been few studies on the relationship between structure and biological activity. Some discussions on the structure-activity relationship were not convincing enough. Finding out the antibacterial sources of isoeugenol-based DHP will improve the synthesis technique of DHP and strengthen the antibacterial properties. They will also provide a new way for the modification and utilization of lignin.

In the present study, the lignin precursor isoeugenol was polymerized to DHP using the bulk method catalyzed by laccase. The obtained DHP was further fractionated with solvents having different solubilizing ability. Moreover, the fractions were further purified by column chromatography to obtain nine compounds. Subsequently, the relationship of antimicrobial activity between structures of the obtained DHP fractions and purified compounds were investigated.

**EXPERIMENTAL**

**Materials**

Isoeugenol (98%) was purchased from Sigma Co., Ltd. (Shanghai, China). Laccase (No. 51003) was from Novazyme Co., Ltd. (Tianjing, China). Its activity was determined to be 1093 IU·mL⁻¹ by the methods of Fukushima and Kirk (1995). All other chemicals were of an analytical grade.
Gram-negative bacterium *E. coli* ATCC 25922 and Gram-positive bacterium *Staphylococcus aureus* CMCC(B) 26003 were obtained from Shanghai Luwei Technology Co., Ltd. (Shanghai, China). Bacterial inoculums were prepared to obtain a bacterial suspension in a nutrient medium (5 mL). The common nutritional agar culture medium was purchased from Aobox Biotechnology Company (Beijing, China) and used for the agar plates. An autoclave was used for sterilization and medium preparation at 121 °C for 20 min.

**Methods**

*Synthesis of DHP*

Isoeugenol (5.0 g) was dissolved in a mixture (1:1 v/v, 50 mL) of ethanol and acetate buffer (0.1 M, pH 5.0), and mixed with laccase (1093 IU·mL⁻¹, 1 mL) with a bulk method. During the reaction, sterile air was bubbled through the mixture, which was maintained in a water bath at 30 °C. After 24 h, the crude product was collected through centrifugation and washed with distilled water. After freeze-drying, the crude product was dissolved in dichloroethane:ethanol (2:1 v/v, 60 mL) *via* stirring at 20 °C for 6 h. The supernatant was collected by centrifugation and DHP was obtained after removing the solvent *in vacuo*.

*Fractionation of DHP*

As shown in Fig. 1, the DHP was sequentially extracted by petroleum ether, diethyl ether, ethanol, and acetone (Li *et al.* 2012). The DHP (4.0 g) was suspended in petroleum ether (b.p. 30 to 60 °C, 200 mL) with magnetic stirring.

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**Fig. 1.** Fractionation procedure of DHP
The petroleum ether fraction (F1) was obtained via centrifugation followed by concentration of the supernatant in a 25.6% yield. This precipitate was further fractionated with diethyl ether, ethanol, and acetone using the same method. Fractions F2, F3, and F4 were obtained with yields of 63.9%, 2.5%, and 1.0%, respectively.

**Molecular weight determination**

A total of 2 mg of each F1, F2, F3, and F4 were dissolved in tetrahydrofuran and filtered through a 0.22-μm membrane. The molecular weights were determined by gel permeation chromatography (GPC) using a Shim-pack GPC-803D column (Shimadzu, Kyoto, Japan) (300 mm × 8 mm). Tetrahydrofuran was applied as an eluent with a flow rate of 0.6 mL·min⁻¹, a column temperature of 30 °C, and an injection volume of 25 μL. Polystyrene was used as standard.

**Evaluation of antimicrobial activity**

The antimicrobial activity of the DHP fractions and isolated compounds was tested using the filter paper agar diffusion method (Carović-Stanko et al. 2010; Muniandy et al. 2014; Choi et al. 2016) according to the zone of inhibition. The bacterial suspension was diluted with sterile saline to 1.5 × 10⁸ CFU·mL⁻¹, and this suspension (200 μL) was uniformly coated onto every agar plate. The four DHP fractions and nine isolated compounds were dissolved in dimethyl sulfoxide (DMSO):normal saline (4:96 v/v, 0.1% polysorbate 80 as dispersant) to obtain a series of solutions with concentration of 5 mg·mL⁻¹. Dried sterile filter papers of 6.00 mm in diameter were immersed in the above solutions for 6 h, then removed and attached onto the agar plates containing bacterium, followed by addition of the corresponding DHP sample solutions (10 μL) onto the surface of the filter papers. After culturing at 37 °C for 24 h, the zone of inhibition was observed and measured.

**Isolation of antimicrobial compounds from the fractions**

The bacteriostatic compounds were further isolated from fractions F1 and F2 by column chromatography (Tan et al. 2011; Xiang 2015). The columns were filled with silica gel of mesh size 100 to 200 and 200 to 300. As shown in Fig. 2, the eluents were acetone:n-hexane (1:9 v/v), acetone:n-hexane (2:3 v/v), and methanol:chloroform (1:20 v/v). Nine compounds were obtained from the eluted solution, which were recorded as Z1, Z2, Z3, Z4, Z5, Z6, Z7, Z8, and Z9, with yields of 62.1%, 2.6%, 1.9%, 11.3%, 1.4%, 1.2%, 9.0%, 0.8%, and 9.7% respectively.

![Fig. 2. Isolation of antimicrobial compounds from the fractions of DHP using column chromatography](image)
Structural analysis of the antimicrobial compounds isolated from DHP

The molecular weights of the compounds were measured using the Agilent 1100 LC/MS (LC-MSD TRAP XCT; Agilent Technologies Inc., Santa Clara, CA, USA) with APCI (+) ion source, 50 to 850 m/z scanning range, 3500 V capillary voltage, nitrogen dryer with a drying temperature of 300 °C, nebulizer temperature of 300 °C, and a dryer flow rate of 5.00 L·min⁻¹. The purified nine compounds (Z₁ to Z₉) were introduced directly into the MSD part to obtain their mass spectroscopy.

The ¹³C-NMR spectrum of the sample was determined at 100.6 MHz with a Varian oneProbe 400 NMR spectrometer (Varian, USA). The sample was placed in a φ5 mm determining tube and dissolved in 0.6 mL CDCl₃ solvent. Pulse delay was 1.75 s with acquisition time of 0.9 s. The sample was scanned approximately 3000 times.

RESULTS AND DISCUSSION

Molecular Weight of DHP

The molecular weights of the four DHP fractions, i.e., F₁, F₂, F₃, and F₄, are shown in Table 1. The weight-average molecular weights (Mₙ) of the F₁, F₂, F₃, and F₄ were 330, 621, 1211, and 3670, respectively. The molecular weights of the fractions increased with the solubility of the solvent. A smaller number-average molecular weight (Mₙ) resulted in a higher low molecular substances content in the fraction. Because the molecular weight of the monomeric compound isoeugenol was 162, the main components of the four fractions were determined to be its dimer, tetramer, heptamer, and dodecamer. The dispersion coefficient (Mₙ/Mₘ) of the four components of F₁, F₂, F₃, and F₄ was approximately 2.60, which indicated that there were many low molecular weight substances in the DHP fractions.

Table 1. Average Molecular Weight of the DHP Fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mₘ (g/mol)</th>
<th>Mₙ (g/mol)</th>
<th>Mₘ/Mₙ</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₁</td>
<td>330±18</td>
<td>126±8</td>
<td>2.62±0.33</td>
</tr>
<tr>
<td>F₂</td>
<td>621±27</td>
<td>237±12</td>
<td>2.62±0.26</td>
</tr>
<tr>
<td>F₃</td>
<td>1211±35</td>
<td>449±21</td>
<td>2.70±0.21</td>
</tr>
<tr>
<td>F₄</td>
<td>3670±73</td>
<td>1579±58</td>
<td>2.32±0.20</td>
</tr>
</tbody>
</table>

Antibacterial Analysis of the Fractions from DHP

The bacteriostatic effects of DHP fractions F₁ to F₄ were evaluated by the size of the zone of inhibition. As shown in Fig. 3, Fig. 4, and Table 2, the inhibitory effects of isoeugenol monomers and solvent on the growth of the two tested bacteria were not obvious.

Fractions F₁ to F₄ had different inhibitory effects on the two test bacteria at the same concentration. The fraction F₁ had obvious inhibitory effects on E. coli and S. aureus, and F₂ had relatively weak inhibitory ability against the both bacteria. Both F₃ and F₄ did not inhibit the growth of the two test bacteria, revealing that molecular weight could have affected the antibacterial properties of the compounds.
Fig. 3. Antibacterial activities of the DHP fractions and isoeugenol against *E. coli*; 
a: F₁, b: F₂, c: F₃, d: F₄, e: isoeugenol, f: black (solvent)

Fig. 4. Antibacterial activities of the DHP fractions and isoeugenol against *S. aureus*; 
a: F₁, b: F₂, c: F₃, d: F₄, e: isoeugenol, f: black (solvent)

Table 2. Zone of Inhibition of DHP Fractions Against the Two Tested Bacteria

<table>
<thead>
<tr>
<th>Tested Bacteria</th>
<th>Zone of Inhibition (mm)</th>
<th>F₁</th>
<th>F₂</th>
<th>F₃</th>
<th>F₄</th>
<th>Isoeugenol</th>
<th>Blank (solvent)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td>8.07±0.13</td>
<td>7.24±0.10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td>9.11±0.08</td>
<td>8.16±0.15</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Structural Analysis of the Isolated Compounds

To understand the structural information of compounds Z₁ to Z₉ isolated from fractions F₁ and F₂, atmospheric pressure chemical ionization mass spectrometry (APCI-MS) was used to analyze the comprehensive molecular weight information of each compound (Evtuguin and Amado 2003; Reale *et al.* 2004). Compared with the starting material isoeugenol, the DHP contained more carboxyl groups, carbonyl groups, and
hydroxyl groups. Considering these oxidized groups and different structural linkages, the average molecular weight of the guaiacyl units in the DHP was calculated as 170.

The compounds Z₁ to Z₄ were eluted from the fraction F₁ and F₂ by a mixture solvent of acetone:n-hexane (1:9 v/v). Figure 5 shows that there was only one peak at m/z 327 [M+H]^+ in the Mass spectrum of Z₁ without corresponding fragment information in the secondary spectrum of m/z 327. This indicated the structure to be relatively stable during mass spectrum detection. The compound Z₁ was assigned as dehydrodiisoeugenol with β-5 structure (Fig. 15, I) (Yang et al. 2010). As shown in Fig. 6, the nuclear magnetic spectrum of Z₁ also confirmed this structure. The molecular ion peak at m/z 329 in the spectrum of Z₂ (Fig. 7) revealed compound II to be a β-5 dimer with 1-CHO and γ'-OH structural substitutions (Fig. 15, II). The ion peak at m/z 315 revealed the structure of 2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methyl-2,3-dihydrobenzofuran-5-carbaldehyde, a dimer fragment with [β-5,1-CHO] structure, due to oxidation of the original side chains of the β-5 substructure by laccase to form carbonyl groups. The presence of a peak at m/z 233 indicated the presence of an ether bond formed by oxidation of a double bond on the side chain of the isoeugenol monomer. As shown in Fig. 8, according to the ion peaks at m/z 203, 327, 343, 463, and 489 in the spectrum of Z₃, this structure was assigned to a β-5 type trimer (Fig. 15, III) with the molecular ion at m/z 489. The molecular weight of Z₄ was at m/z 651 in Fig. 9, which corresponded to the tetramer structure of [(β-5)-(β-5)-(β-5)], as shown in Fig. 15 (IV).

The compound Z₅ to Z₇ were eluted from the fraction F₂ by a mixture solvent of acetone:n-hexane (2:3 v/v). The molecular signal at m/z 671 in the mass spectrum of the Z₅ compound (Fig. 10) was from the structure of the tetramer [(β-5)-(β-O-4)-(β-5), γ'-CH₂OH, α'-CHO] (Fig. 15, V). There was a weak fragment signal at m/z 509, which was assigned to 2-[4-[2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl-ethoxy]-3-methoxy-phenyl]-3-hydroxymethyl-7-methoxy-2,3-dihydrobenzofuran-5-carbaldehyde, a trimer with [(β-5)-(β-O-4), γ'-CH₂OH, α'-CHO]. The signal at m/z 329 could have been assigned to 2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydrobenzofuran-5-carbaldehyde, a dimer fragment with β-5 structure from the cleavage of a β-O-4 subunit and a β-5 subunit in Z₅. An ion peak appeared at m/z 233. This was from the monomeric fragment formed by cleavage of the aryl ether bond and β-5 linkage of Z₅. From the molecular ion at m/z 731 in the MS spectrum of Z₆ (Fig. 11), this compound was determined to be a tetramer with [(β-O-4)-(β-5)-(β-5), α'-COOH] structure (Fig. 15, VI). A molecular ion was observed at m/z 831 in the spectrum of Z₇ (Fig. 12) and assigned to a pentamer structure with [(β-5)-(5-5)-(β-5)-(β-O-4)-(β-O-4)] (Fig. 15, VII). A fragment signal at m/z 500 was from the 2-(6,2'-dihydroxy-5,3'-dimethoxy-5'-propyl-biphenyl-3-yl)-7-methoxy-3-methyl-2,3-dihydrobenzofuran-5-carbaldehyde, which was a [(β-5)-(5-5), α'-CHO] structure. The peak at m/z 332 was assigned to the dimer fragment with [(β-O-4), α'-CHO] structure, i.e. 4-[2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl-ethoxy]-3-methoxy-benzaldehyde, which was formed by cleavage of another β-O-4 subunit.

The compounds Z₈ and Z₉ were eluted by a mixture of high swelling solvent of chloroform and high polarity solvent of methanol. The molecular ion peak at m/z 651 in the mass spectrum of Z₈ in Fig. 13 was from the Z₈, [(β-5)-(α-O-4)-(5-5)], as shown in Fig. 15 (VIII). The peak at m/z 489 was from the fragment of 4-(4,9-dimethoxy-7-methyl-2,11-dipropenyl-6,7-dihydro-5,8-dioxa-dibenzo[a,c]cycloocten-6-yl)-2-methoxyphenol, which was a trimer with [(α-O-4)-(5-5)] structure derived from the elimination of a β-5 subunit. The peak at m/z 343 was 4-[5-(1-hydroxy-propyl)-7-methoxy-3-methyl-2,3-
The molecular ion of Z9 appeared at m/z 671 in the MS spectrum of compound Z9 in Fig. 14, which had the structure of a tetramer, [(β-5)-(α-O-4)-(5-5), α-CHO, γ-CH2OH×2] (Fig. 15, IX). The related fragment peak at m/z 326 was assigned to 2-methoxy-4-(7-methoxy-3-methyl-5-propyl-2,3-dihydro-benzofuran-2-yl)-phenol, a dimer structure of β-5.

Fig. 5. Mass spectrum of compound Z1;
Legend: m/z 327: 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2-yl)-phenol, (dehydrodiisoeugenol with β-5)

Fig. 6. $^{13}$C-NMR spectrum of the compound Z1;
Legend: The main signals: δ146.71 (C3), δ146.53 (C4), δ145.78 (C4'), δ144.12 (C3'), δ133.26 (C5'), δ132.18 (C1), δ132.02 (C1'), δ130.92 (Cα'), δ123.47 (Cβ'), δ119.94 (C6), δ114.13 (C5), δ113.30 (C6'), δ109.18 (C2'), δ108.94 (C2), δ93.79 (Cα), δ55.94 (C7 and C7'), δ45.61 (Cβ), δ 18.41 (Cγ), δ17.54 (Cγ')
Fig. 7. Mass spectrum of the compound Z2:
Legend: m/z 233.6: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na⁺], {α-OC₅H₅[Na⁺]}; m/z 315.6: 2-
(4-hydroxy-3-methoxy-phenyl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-carbaldehyde, (β-5,
1-CHO); m/z 329.5: 2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-
benzofuran-5-carbaldehyde, (β-5 dimer with 1-CHO, γ'-OH)

Fig. 8. Mass spectrum of the compound Z3:
Legend: m/z 203.5: 4-(1-hydroxy-propyl)-2-methoxy-phenol [Na⁺], {α-OH [Na⁺]}; m/z 327.2: 2-
methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2-yl)-phenol, (β-5); m/z 343.1: 4-
[1-hydroxy-2-(2-methoxy-4-propenyl-phenoxy)-propyl]-2-methoxy-phenol, (β-0-4, α-OH);
m/z 489.1: 4-(7,7'-dimethoxy-3,3'-dimethyl-5-propenyl-2,3,2',3'-tetrahydro-[2,5]bibenzofuranyl-2'-
yl)-2-methoxy-phenol, (β-5)-(β-5)

Fig. 9. Mass spectrum of the compound Z4:
Legend: m/z 203.5: 4-(1-hydroxy-propyl)-2-methoxy-phenol [Na+], {α-OH [Na+]; m/z 343.0: 4-[1-hydroxy-2-(2-methoxy-4-propenyl-phenoxo)-propyl]-2-methoxy-phenol, (β-O-4, α-OH); m/z 409.1: 3-ethoxy-3-(4-hydroxy-3-methoxy-phenyl)-2-(2-methoxy-4-propenyl-phenoxo) propionaldehyde, (β-O-4, α-C2H5, β-CHO); m/z 489.1: 4-(7,7’-dimethoxy-3,3’-dimethyl-5-propenyl-2,3,2’,3’-tetrahydro-[2,5’]bibenzofuranyl-2’,yl)-2-methoxy-phenol, (β-5)-(β-5); m/z 651.0: 2-methoxy-4-(7,7’,7’-trimethoxy-3,3’,3’-trimethyl-5-propenyl-2,3,2’,3’,3’-hexahydro-[2,5’,2’,5’]terbenzofuran-2’’-yl)-phenol, (β-5)-(β-5)-(β-5)

Fig. 10. Mass spectrum of the compound Z5;
Legend: m/z 233.6: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na+], {α-OC2H5[Na+]; m/z 329.8: 2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-carbaldehyde, (β-5, α-CHO); m/z 377.8: 2-(4-hydroxy-3-methoxy-phenyl)-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, (β-5, γ-OH); m/z 509.8: 2-[4-(2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl-ethoxy)-3-methoxy-phenyl]-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-carbaldehyde, [(β-5)-(β-O-4), γ-CH2OH, 1-CHO]; m/z 564.3: 2-[4-(2-ethoxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl-ethoxy)-3-methoxy-phenyl]-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, [(β-O-4)-(β-5), α-OC2H5, β-CHO]; m/z 670.9: 2-[4-(2-hydroxy-2-[2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-yl]-1-methyl-ethoxy)-3-methoxy-phenyl]-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-carbaldehyde, [(β-5)-(β-O-4)-(β-5), γ-CH2OH, α-CHO]

**Fig. 11.** Mass spectrum of the compound Z₆;
Legend: m/z 233.4: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na⁺], {α-OC₂H₅[Na⁺]}; m/z 334.5: 3-hydroxy-2,3-bis-(4-hydroxy-3-methoxy-phenyl)-propionic acid, [(β-O, α-COOH); m/z 378.1: 2-(4-hydroxy-3-methoxy-phenyl)-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, [(β-5, β-CHO, γ'-OH]; m/z 563.3: 3-(2-[4-{1-carboxy-2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-ethyl}-3-methoxy-phenyl]-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-yl)-acrylic acid, [(β-O-(β-5), α-COOH]; m/z 731.9: 2'-(4-[1-hydroxy-(4-hydroxy-3-methoxy-phenyl)-methyl]-2-oxo-propoxy]-3-methoxy-phenyl]-7,7' dimethoxy-3'-methyl-2,3,2',3'-tetrahydro-[2,5]bibenzofuranyl-3,5-dicarboxylic acid, [(β-O-(β-5)-(β-5), α-COOH]

**Fig. 12.** Mass spectrum of the compound Z₇;
Legend: m/z 233.2: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na⁺], {α-OC₂H₅[Na⁺]}; m/z 331.5: 4-[2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl-ethoxy]-3-methoxy-benzaldehyde, (β-O, α'-CHO); m/z 379.1: 2-(4-hydroxy-3-methoxy-phenyl)-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, [(β-5, β-CHO, γ'-OH]; m/z 500.6: 2-(6,2''-dihydroxy-5,3'' dimethoxy-5' propyl-biphenyl-3-yl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-carbaldehyde, [(β-5)-(5-5), α-COOH]; m/z 564.2: 2-[4-[2-ethoxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl ethoxy]-3-methoxy-phenyl]-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, [(β-O-(β-5), α-COOH]; m/z 831.2: 2-(6,2''-dihydroxy-5,3'' dimethoxy-5' [2-(2 methoxy-4-(2-[2-methoxy-4-(3-oxo-propenyl)-phenoxyl-propionyl]-phenoxy)-propyl]-biphenyl-3 yl]-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-carbaldehyde, [(β-5)-(5-5)-(β-5)-(β-O-(α-O, β-CHO])

Fig. 13. Mass spectrum of the compound Z₃;
Legend: m/z 203.9: 4-(1-hydroxy-propyl)-2-methoxy-phenol [Na⁺], [α-OH][Na⁺]; m/z 343.3: 4-[5-(1-hydroxy-propyl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-2-yl]-2-methoxy-phenol, (β-5, α-OH); m/z 489.3: 4-(4,9-dimethoxy-7-methyl-2,11-dipropenyl-6,7-dihydro-5,8-dioxadia-benzo[a,c]cycloocten-6-yl)-2-methoxy-phenol, [α-O-4]-4,4-dimethoxy-7-methyl-2,11-dipropenyl-6,7-dihydro-5,8-dioxadia-benzo[a,c]cycloocten-6-yl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-2-yl]-2-methoxy-phenol, ([β-5)-(α-O-4)-(5-5)]

Fig. 14. Mass spectrum of the compound Z₅;
Legend: m/z 182.4: 4-(3-hydroxy-propyl)-2-methoxy-phenol, (γ-OH); m/z 326.3: 2-methoxy-4-(7-methoxy-3-methyl-5-propyl-2,3-dihydro-benzofuran-2-yl)-phenol, (β-5); m/z 378.2: 1-[2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-yl]-propane-1,3-diol, (β-5, γ-OH, α-OH, γ'-OH); m/z 490.4: 7-(4-hydroxy-3-methoxy-phenyl)-6-hydroxymethyl-4,9-dimethoxy-11-propenyl-6,7-dihydro-5,8-dioxadia-benzo[a,c]cyclooctene-2-carbaldehyde, [(α-O-4)-(5-5), γ-OH]; m/z 507.5: 4-[5-[3-hydroxy-1-(2-methoxy-4-propenyl-phenoxy)-propyl]-7-methoxy-3-methyl-2,3-dihydro-benzofuran-2-yl]-2-methoxy-phenol, [(β-5)-(α-O-4), γ-OH, γ'-OH]; m/z 671.1: 7-[2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-yl]-6-hydroxymethyl-4,9-dimethoxy-11-propenyl-6,7-dihydro-5,8-dioxadia-benzo[a,c]cyclooctene-2-carbaldehyde, [(β-5)-(α-O-4)-(5-5), α-CHO, γ-CH₂OH×2]

Fig. 15. Chemical structures of the compounds Z₁ to Z₉

Antibacterial Analysis of the Isolated Compounds Z₁ to Z₉

As shown in Fig. 16 and Fig. 18, of the compounds Z₁ to Z₉, only compounds Z₁ and Z₂ from the F₁ fraction had an obvious inhibitory effect on *E. coli* in the antibacterial test. Considering the relatively week inhibitory effect of F₂ (Z₃ to Z₉) on *E. coli* as shown in Table 2, it could be concluded that some bioactive compounds had not been isolated from the F₂ fraction.
Although the antibacterial property of isoeugenol was not obvious, as shown in the Table 2, the dimer of the DHP (dehydrodiisoeugenol) showed significant activity. It indicated that the dimers with a β-5 structure could effectively inhibit the growth of *E. coli*. This was consistent with the study of Hattori *et al.* (1986). They found that dehydrodiisoeugenol and 5′-methoxydehydrodiisoeugenol were the major antibacterial principles of extracted fractionations from *Myristica fragrans*. These were attributed to their inhibitory action against glucosyltransferase of bacteria and lead to the loss of adhesive ability of the bacteria. Senioa *et al.* (2018) also found that the minimum inhibitory concentrations (MICs) of *Galium aparine* L. infusions and hydromethanolic extract containing phenylcoumaran on *E. coli* and *S. aureus* were 3.75 to 30 mg/mL and 1.85 to 10 mg/mL, respectively. Zhang *et al.* (1984) synthesized 10 coumaran derivatives and found some compounds had obvious prophylactic and curative activities against infection of *Schistosomiasis japonica* in mice. Therefore, the strong bioactivity of coumaran structure was demonstrated. The diameter of the inhibition zones of **Z**₁ was...
larger than that of $Z_2$. The relatively weak inhibitory ability of $Z_2$ may have been due to the fact that the double bond of the isoeugenol side chain was oxidized to an aldehyde. This result was in good agreement with that of Jay and Rivers (1984) and Hyldgaard et al (2015). They found the MIC of vanillin was much higher than that of isoeugenol against E. coli.

![Image of antibacterial activities of compounds Z1 to Z9]

**Fig. 17.** Antibacterial activities of the compounds Z1 to Z9 isolated from the F1 and F2 fractions against S. aureus

Furthermore, all nine compounds had a relatively strong inhibitory effect on S. aureus, as shown in Figs. 17 and 18. This may have been related to their β-5 structure. The β-5 dimer Z1 had a strongest inhibitory effect on S. aureus with an average diameter of 12.08 mm. Figures 17 and 18 showed that although Z2 inhibited the growth of S. aureus and produced a clear zone of inhibition, the diameter of the inhibition zone was smaller than that of Z1. This also indicated that the aldehyde group decreased the antibacterial properties of the substance, with the carbon-carbon double bond of the side chain contributing more to the bacteriostatic activity than the aldehyde group as stated above. As shown in Fig. 18, the compounds Z7, Z8, and Z9 had a relatively weak effect on S. aureus. This may have been due to the 5-5 condensed structure of these compounds. In general, the comparison of the inhibition zone of Z1 to Z9 against the two test bacteria showed their antibacterial activity against E. coli to be weaker than against S. aureus. In summary, the inhibitory effect of Z1 to Z9 on Gram-positive bacteria was better than on
Gram-negative bacteria. Moreover, it was also found that the compound Z₁ (dehydrodiisoeugenol) had the greatest inhibitory effect on the two test bacteria.

![Graph showing inhibition zone](image)

**Fig. 18.** Zone of inhibition of the compounds Z₁ to Z₉ isolated from the F₁ and F₂ fractions against *E. coli* and *S. aureus*; Legend: The diameter of inhibition of the samples with no antibacterial activity was subtracted the diameter of the filter paper (6.00 mm)

**CONCLUSIONS**

1. The four fractions of DHP from isoeugenol, *i.e.*, F₁, F₂, F₃, and F₄ showed remarkable differences in their growth inhibition of *E. coli* and *S. aureus*, with the F₁ and F₂ fractions showing strong antibacterial activity against both species.

2. The bacteriostatic compounds were further separated sequentially by column chromatography from the F₁ and F₂ fractions with eluents of acetone:n-hexane (1:9 v/v), acetone:n-hexane (2:3 v/v), and methanol:chloroform (1:20 v/v). Nine compounds (Z₁-Z₉) were obtained from the eluate. APCI-MS spectrometry was applied to determine the chemical structure of the nine compounds, which were found to be dimers, trimers, tetramers, and pentamers with β-5, β-O-4, and 5-5 substructures, and with partial side chain oxidation to a α-aldehyde via dehydropolymerization.

3. Antibacterial experiments showed that dimers (Z₁ and Z₂) could inhibit bacteria such as *S. aureus* and *E. coli*. The Z₃ to Z₉ compounds could only inhibit *S. aureus* but had no inhibitory effect against *E. coli*. 

4. Considering the structure-effect relationship, the aldehyde groups and the condensed 5-5 structure may decrease the antibacterial properties of DHP. The formation of the aldehyde groups during the synthesis of DHP catalyzed by laccase weakened its antibacterial properties. However, the formation of the β-5 structure may have been related to the antibacterial ability of DHP.

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