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 (F_1), diethyl ether-soluble fraction (F_2), ethanol-soluble fraction (F_3), and acetone-soluble fraction (F_4). The results of antibacterial experiments showed that only F_1 and F_2 could effectively inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. Furthermore, nine compounds (Z_1 to Z_9) were obtained *via* the column chromatographic separation from F_1 and F_2. Mass spectrum analysis results showed that all of these compounds contained a β-5 structure. Antibacterial experiments showed that dimers (Z_1 and Z_2) could inhibit both *S. aureus* and *E. coli*. The trimers, tetramers, and pentamers (Z_3 to Z_9) 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 $IU \cdot mL^{-1}$ 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 ℃ 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.

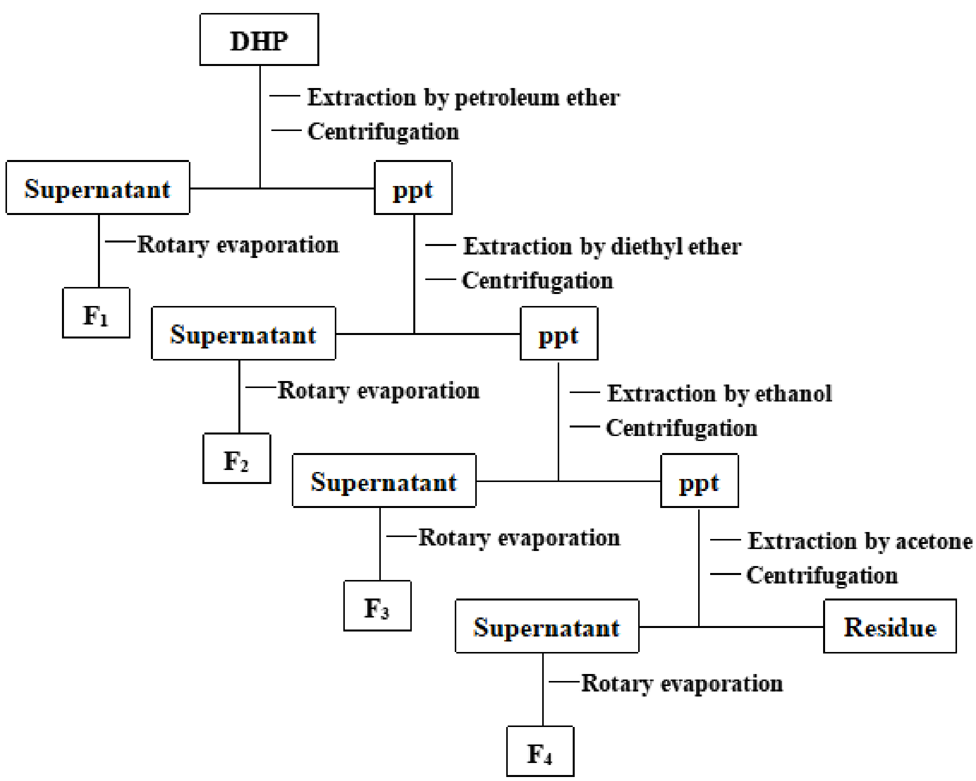

Fig. 1. Fractionation procedure of DHP

The petroleum ether fraction (F_1) 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 F_2, F_3, and F_4 were obtained with yields of 63.9%, 2.5%, and 1.0%, respectively.

Molecular weight determination

A total of 2 mg of each F_1, F_2, F_3, and F_4 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 × 10^8 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 F_1 and F_2 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 Z_1, Z_2, Z_3, Z_4, Z_5, Z_6, Z_7, Z_8, and Z_9, with yields of 62.1%, 2.6%, 1.9%, 11.3%, 1.4%, 1.2%, 9.0%, 0.8%, and 9.7% respectively.

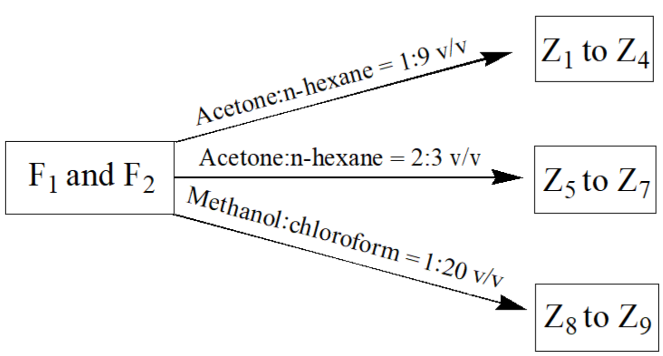

Fig. 2. Isolation of antimicrobial compounds from the fractions of DHP using column chromatography

Structural analysis of the of 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 ℃, nebulizer temperature of 300 ℃, and a dryer flow rate of 5.00 $L \cdot min^{-1}$. The purified nine compounds (Z_1 to Z_9) were introduced directly into the MSD part to obtain their mass spectroscopy.

The ^{13}C-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 $CDCl_3$ 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.*, F_1, F_2, F_3, and F_4, are shown in Table 1. The weight-average molecular weights (M_w) of the F_1, F_2, F_3, and F_4 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 (M_n) 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 (M_w/M_n) of the four components of F_1, F_2, F_3, and F_4 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

Fraction	M_w (g/mol)	M_n (g/mol)	M_w/M_n
F_1	330±18	126±8	2.62±0.33
F_2	621±27	237±12	2.62±0.26
F_3	1211±35	449±21	2.70±0.21
F_4	3670±73	1579±58	2.32±0.20

Antibacterial Analysis of the Fractions from DHP

The bacteriostatic effects of DHP fractions F_1 to F_4 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 F_1 to F_4 had different inhibitory effects on the two test bacteria at the same concentration. The fraction F_1 had obvious inhibitory effects on *E. coli* and *S. aureus*, and F_2 had relatively weak inhibitory ability against the both bacteria. Both F_3 and F_4 did not inhibit the growth of the two test bacteria, revealing that molecular weight could have affected the antibacterial properties of the compounds.

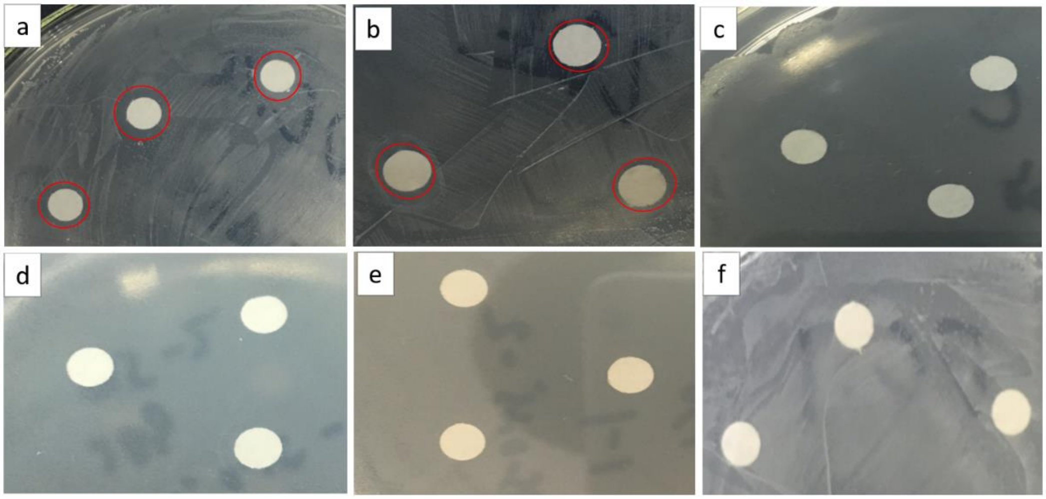

Fig. 3. Antibacterial activities of the DHP fractions and isoeugenol against *E. coli*;
a: F_1 , b: F_2, c: F_3, d: F_4, e: isoeugenol, f: black (solvent)

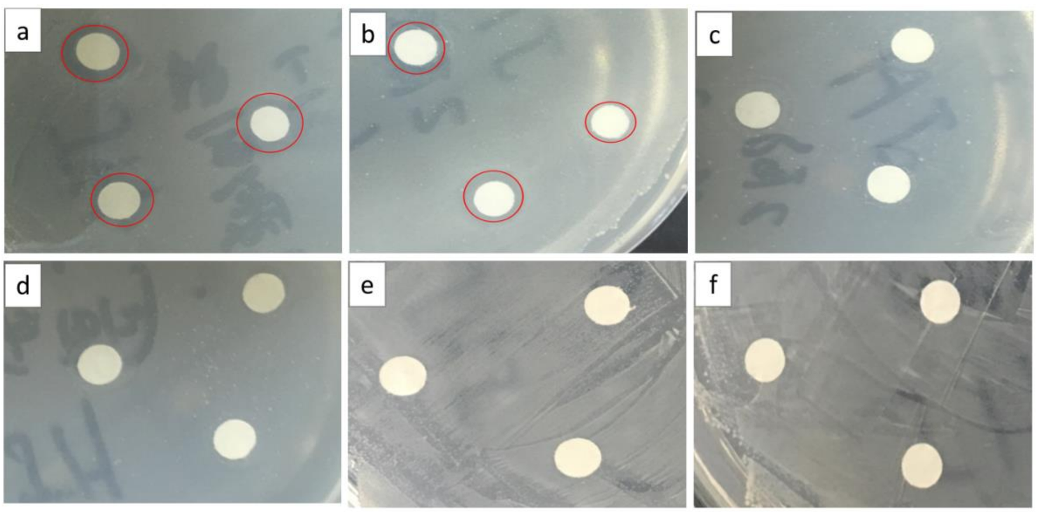

Fig. 4. Antibacterial activities of the DHP fractions and isoeugenol against *S. aureus*;
a: F_1 , b: F_2, c: F_3, d: F_4, e: isoeugenol, f: black (solvent)

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

Tested Bacteria	Zone of Inhibition (mm)					
	F_1	F_2	F_3	F_4	Iso-eugenol	Blank (solvent)
E. coli	8.07±0.13	7.24±0.10	—	—	—	—
S. aureus	9.11±0.08	8.16±0.15	—	—	—	—

Structural Analysis of the Isolated Compounds

To understand the structural information of compounds Z_1 to Z_9 isolated from fractions F_1 and F_2, 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 Z_1 to Z_4 were eluted from the fraction F_1 and F_2 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 Z_1 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 Z_1 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 Z_1 also confirmed this structure. The molecular ion peak at *m/z* 329 in the spectrum of Z_2 (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 Z_3, 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 Z_4 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 Z_5 to Z_7 were eluted from the fraction F_2 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 Z_5 compound (Fig. 10) was from the structure of the tetramer [(β-5)-(β-O-4)-(β-5), γ-CH_2OH, 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-dihydro-benzofuran-5-carbaldehyde, a trimer with [(β-5)-(β-O-4), γ-CH_2OH, 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-dihydro-benzofuran-5-carbaldehyde, a dimer fragment with β-5 structure from the cleavage of a β-O-4 subunit and a β-5 subunit in Z_5. 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 Z_5. From the molecular ion at *m/z* 731 in the MS spectrum of Z_6 (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 Z_7 (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-dihydro-benzofuran-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 Z_8 and Z_9 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 Z_8 in Fig. 13 was from the Z_8, [(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-

dihydro-benzofuran-2-yl]-2-methoxy-phenol, [(β-5)], which was from the fragment formed by the cleavage of α-O-4 linkages (Kang *et al.* 2012; Izumi and Kuroda 1997). The molecular ion of Z_9 appeared at *m/z* 671 in the MS spectrum of compound Z_9 in Fig. 14, which had the structure of a tetramer, [(β-5)-(α-O-4)-(5-5), α-CHO, γ-CH_2OH×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.

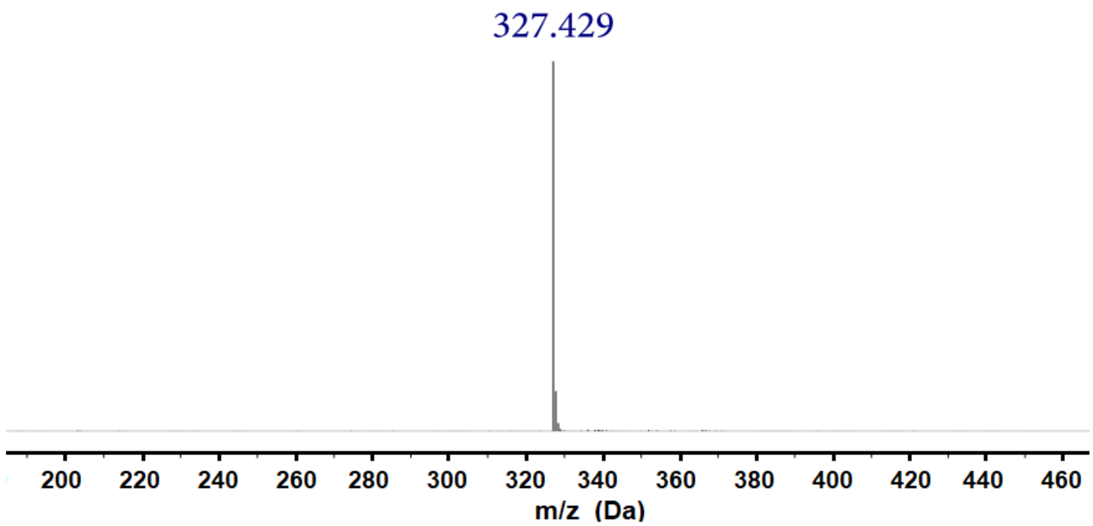

Fig. 5. Mass spectrum of compound Z_1;
Legend: *m/z* 327: 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2-yl)-phenol, (dehydrodiisoeugenol with β-5)

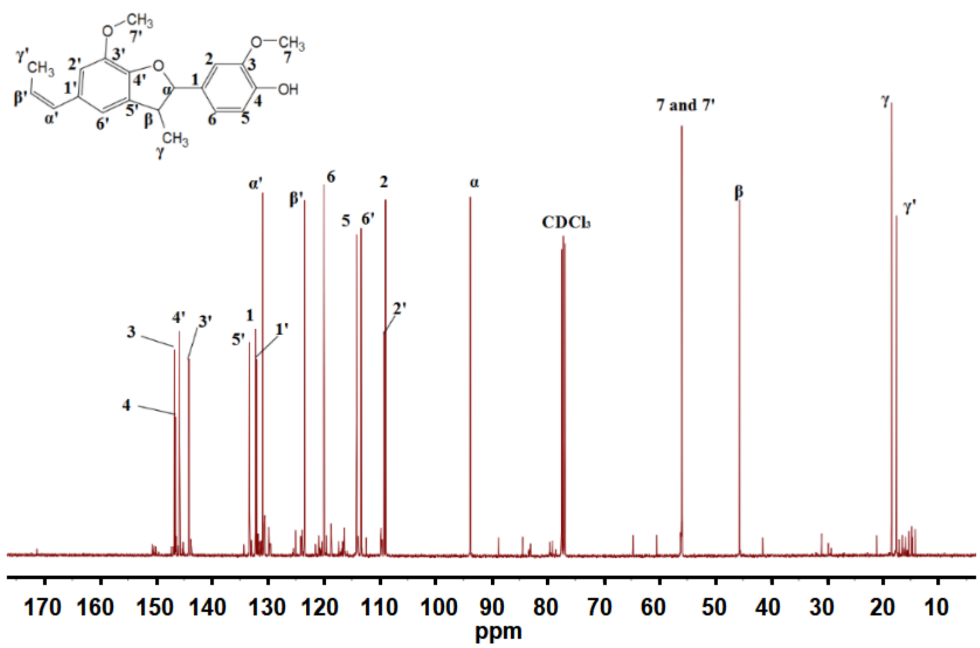

Fig. 6. ^{13}C-NMR spectrum of the compound Z_1;
Legend: The main signals: *δ*146.71 (C3), *δ*146.53 (C4), *δ*145.78 (C4'), *δ*144.12 (C3'), *δ*133.26 (C5'), *δ*132.18 (C1), *δ*132.02 (C1'), *δ*130.92 (C*α*'), *δ*123.47 (C*β*'), *δ*119.94 (C6), *δ*114.13 (C5), *δ*113.30 (C6'), *δ*109.18 (C2'), *δ*108.94 (C2), *δ*93.79 (C*α*), *δ*55.94 (C7 and C7'), *δ*45.61 (C*β*), *δ* 18.41 (C*γ*), *δ*17.54 (C*γ*')

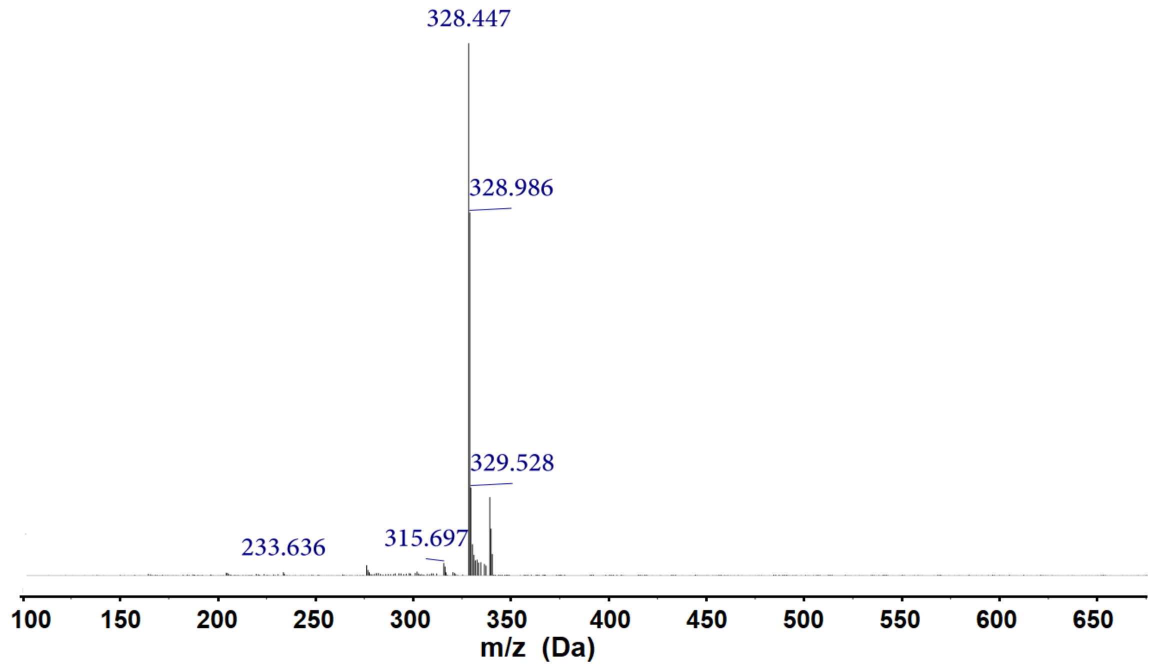

Fig. 7. Mass spectrum of the compound Z_2;
Legend: *m/z* 233.6: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na^+], {α-OC_2H_5[Na^+]}; *m/z* 315.6: 2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-carbaldehyde, (β-5, 1-CHO); *m/z* 329.5: 2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-carbaldehyde, (β-5 dimer with 1-CHO, *γ*'-OH)

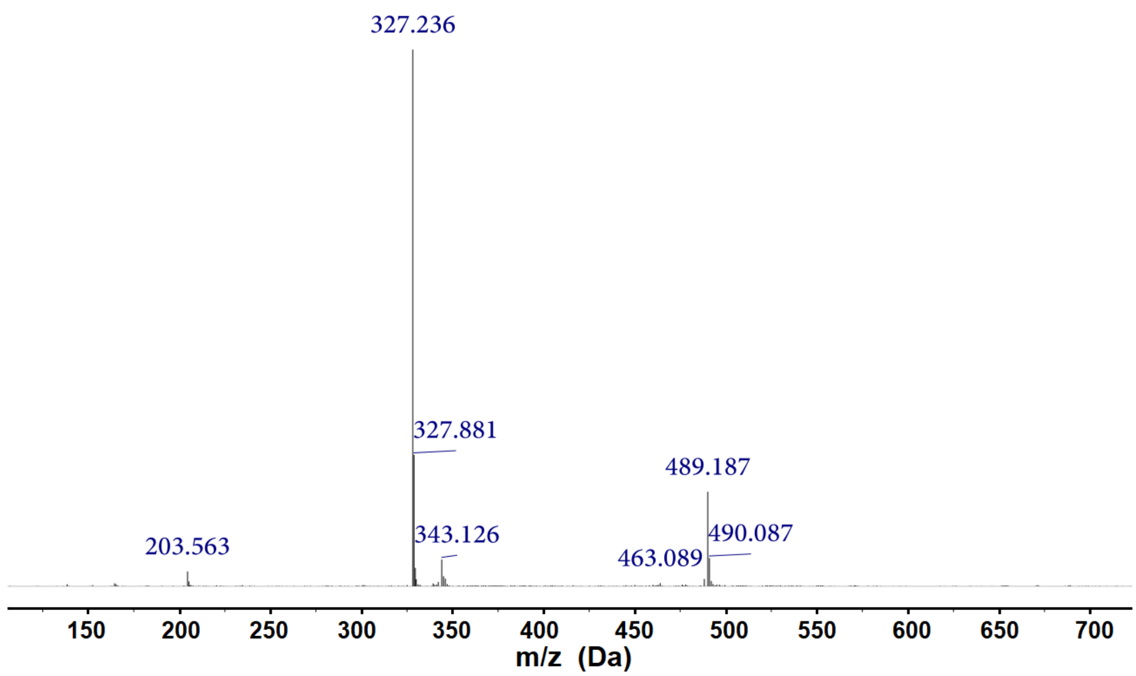

Fig. 8. Mass spectrum of the compound Z_3;
Legend: *m/z* 203.5: 4-(1-hydroxy-propyl)-2-methoxy-phenol [Na^+], {α-OH [Na^+]}; *m/z* 327.2: 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2-yl)-phenol, (β-5); *m/z* 343.1: 4-[1-hydroxy-2-(2-methoxy-4-propenyl-phenoxy)-propyl]-2-methoxy-phenol, (β-O-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)

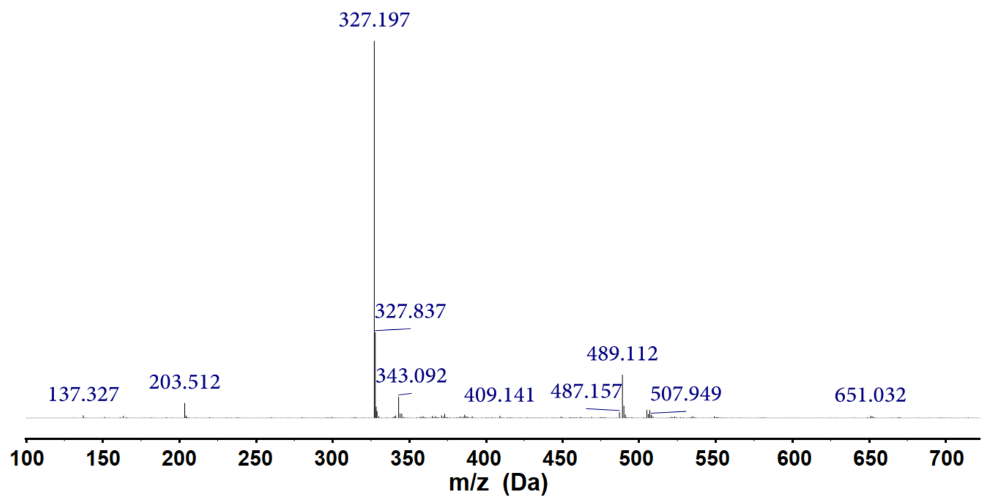

Fig. 9. Mass spectrum of the compound Z_4;
Legend: *m/z* 203.5: 4-(1-hydroxy-propyl)-2-methoxy-phenol [Na^+], {α-OH [Na^+]}; *m/z* 343.0: 4-[1-hydroxy-2-(2-methoxy-4-propenyl-phenoxy)-propyl]-2-methoxy-phenol, (β-O-4, α-OH); *m/z* 409.1: 3-ethoxy-3-(4-hydroxy-3-methoxy-phenyl)-2-(2-methoxy-4-propenyl-phenoxy) propionaldehyde, (β-O-4, α-C_2H_5, 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',2'',3''-hexahydro-[2,5';2',5'']terbenzofuran-2''-yl)-phenol, (β-5)-(β-5)-(β-5)

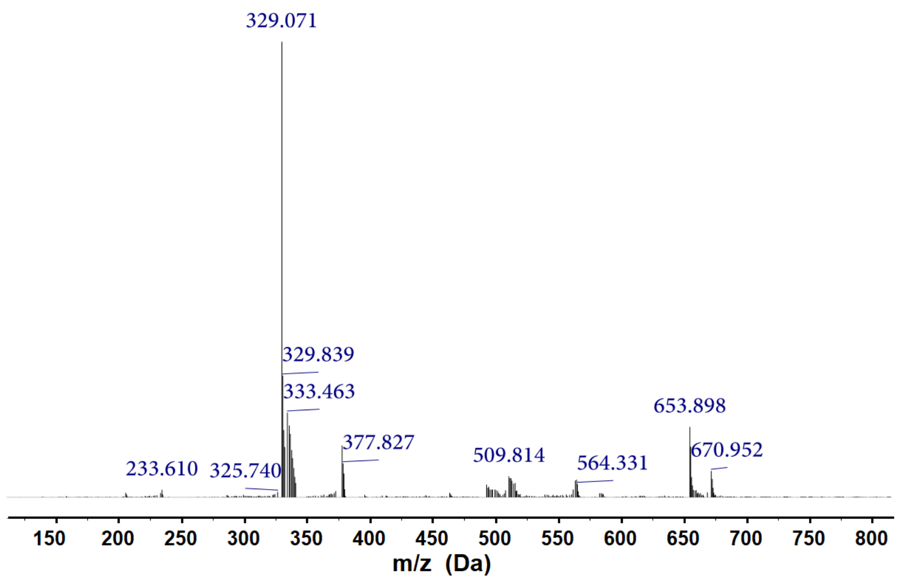

Fig. 10. Mass spectrum of the compound Z_5;
Legend: *m/z* 233.6: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na^+], {α-OC_2H_5[Na^+]}; *m/z* 329.8: 2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-carbaldehyde, (β-5, α-CHO); *m/z* 377.8: 2-(4-hydroxy-3-methoxy-phenyl)-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, (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-CH_2OH, 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-OC_2H_5, 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-CH_2OH, α'-CHO]

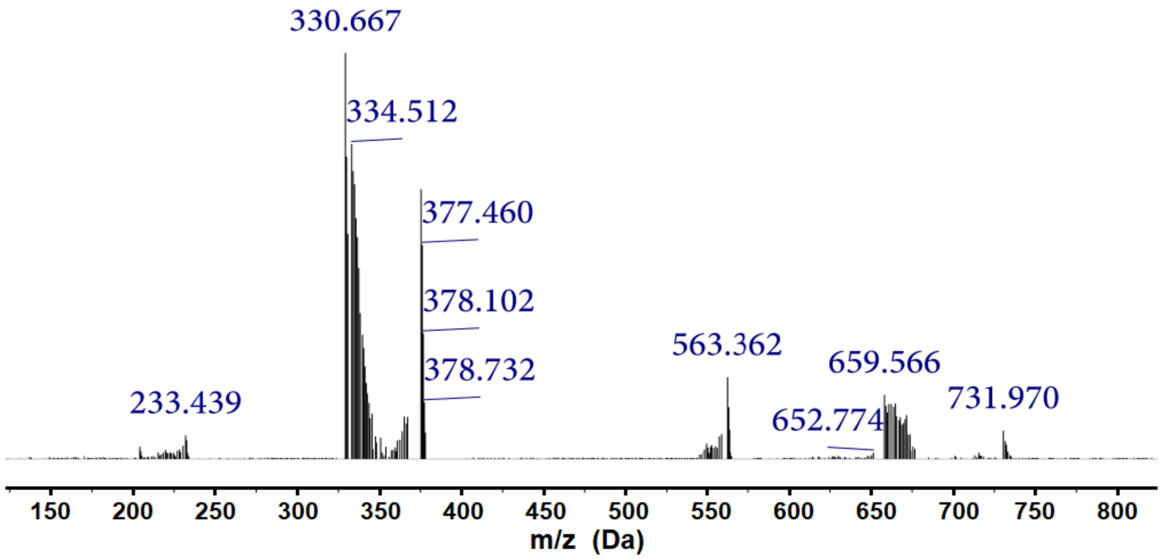

Fig. 11. Mass spectrum of the compound Z_6;
Legend: *m/z* 233.4: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na^+], {α-OC_2H_5[Na^+]}; *m/z* 334.5: 3-hydroxy-2,3-bis-(4-hydroxy-3-methoxy-phenyl)-propionic acid, [(β-O-4), α-COOH]; *m/z* 378.1: 2-(4-hydroxy-3-methoxy-phenyl)-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, [(β-5), β-CHO ,γ'-OH]; *m/z* 563.3: 3-(2-{4-[1-carboxy-2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-ethoxy]-3-methoxy-phenyl}-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-yl)-acrylic acid, [(β-O-4)-(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-4)-(β-5)-(β-5), α-COOH]

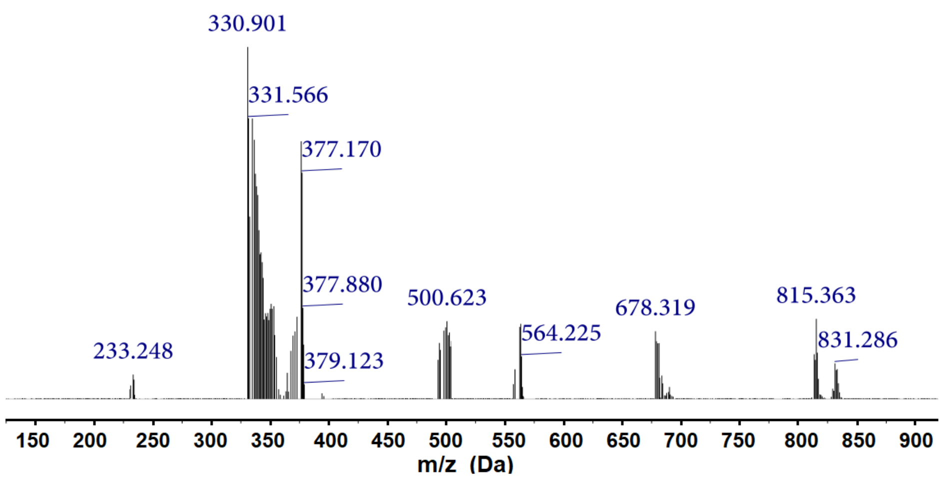

Fig. 12. Mass spectrum of the compound Z_7;
Legend: *m/z* 233.2: 4-(1-ethoxy-propyl)-2-methoxy-phenol [Na^+], {α-OC_2H_5[Na^+]}; *m/z* 331.5: 4-[2-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-1-methyl-ethoxy]-3-methoxy-benzaldehyde, (β-O-4, α'-CHO); *m/z* 379.1: 2-(4-hydroxy-3-methoxy-phenyl)-5-(3-hydroxy-propenyl)-7-methoxy-2,3-dihydro-benzofuran-3-carbaldehyde, [(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-CHO]; *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-4)-(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)-phenoxy]-propionyl}-phenoxy)-propyl]-biphenyl-3-yl}-7-methoxy-3-methyl-2,3-dihydro-benzofuran-5-carbaldehyde, [(β-5)-(5-5)-(β-5)-(β-O-4)-(β-O-4), β-CHO]

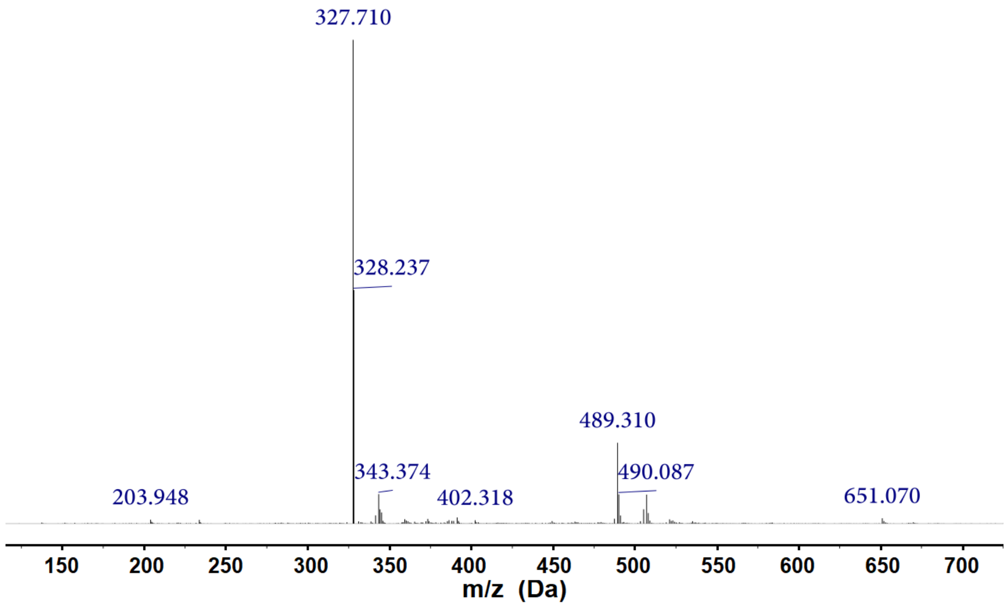

Fig. 13. Mass spectrum of the compound Z_8;
Legend: *m/z* 203.9: 4-(1-hydroxy-propyl)-2-methoxy-phenol [Na^+], {α-OH[Na^+]}; *m/z* 343.3: 4-[5-(1-hydroxy-propyl)-7-methoxy-3-methyl-2,3-dihydro-benzofuran-2-yl]-2-methoxy-phenol, (β-5, α-OH); *m/z* 489.3: 4-(4,9-dimethoxy-7-methyl-2,11-dipropenyl-6,7-dihydro-5,8-dioxa-dibenzo[a,c] cycloocten-6-yl)-2-methoxy-phenol, [(α-O-4)-(5-5)]; *m/z* 651.1: 4-[5-(4,9-dimethoxy-7-methyl-2,11-dipropenyl-6,7-dihydro-5,8-dioxa-dibenzo[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)]

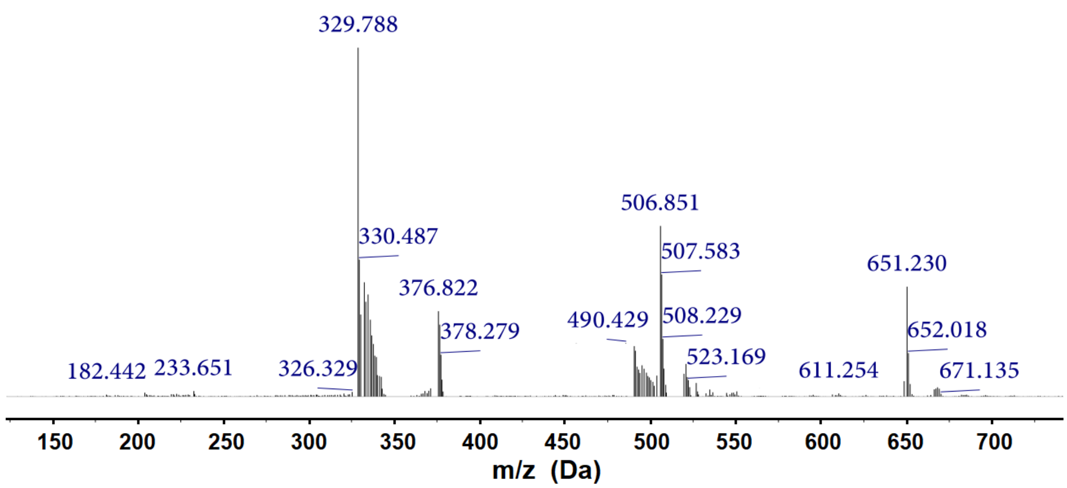

Fig. 14. Mass spectrum of the compound Z_9;
Legend: *m/z* 182.4: 4-(3-hydroxy-propyl)-2-methoxy-phenol, (γ-OH); *m/z* 326.3: 2-methoxy-4-(7-methoxy-3-methyl-5-propyl-2,3-dihydro-benzofuran-2-yl)-phenol, (β-5); *m/z* 378.2: 1-[2-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-benzofuran-5-yl]-propane-1,3-diol, (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-dioxa-dibenzo[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-dioxa-dibenzo[a,c]cyclooctene-2-carbaldehyde, [(β-5)-(α-O-4)-(5-5), α-CHO, γ-CH_2OH×2]

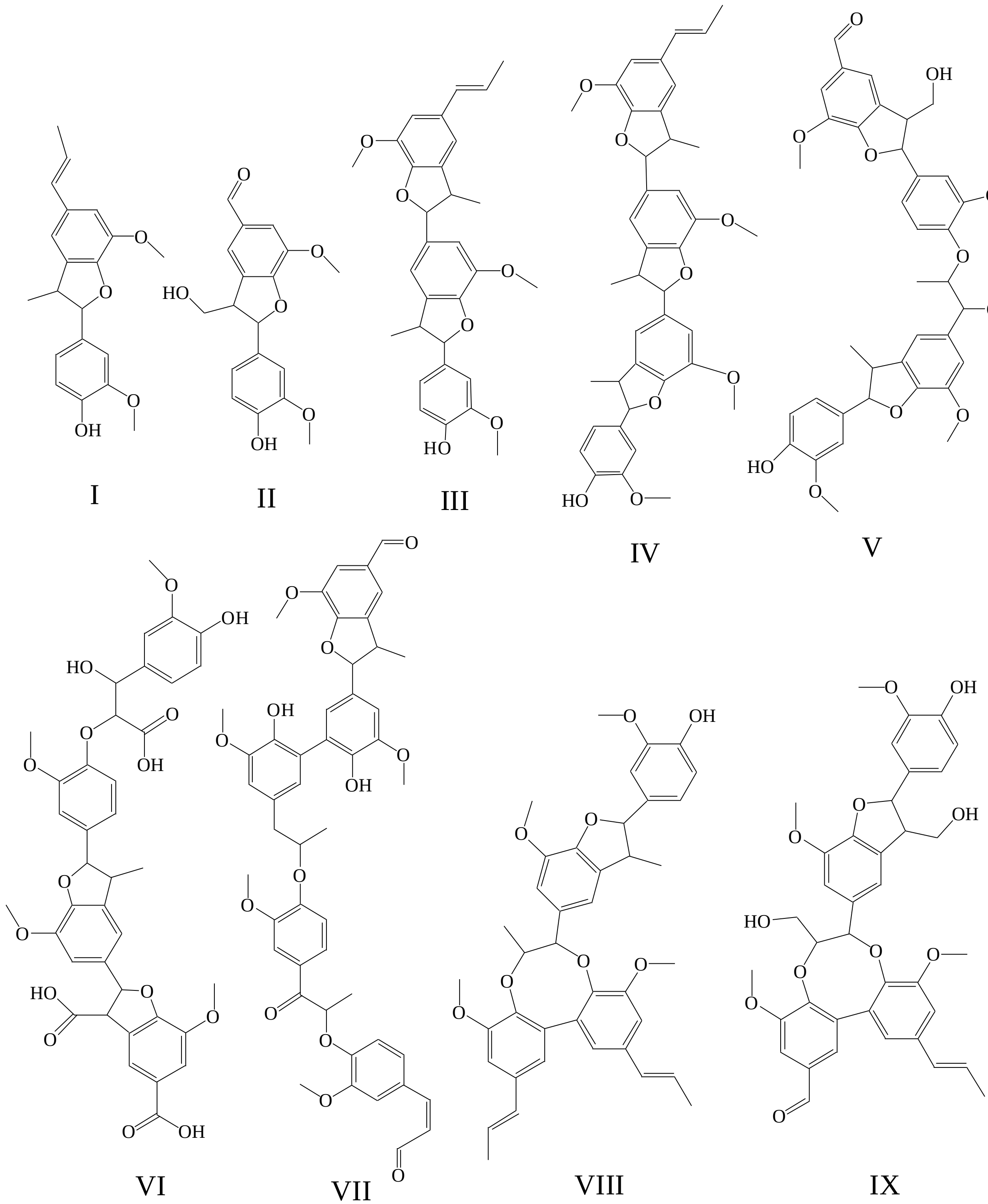

Fig. 15. Chemical structures of the compounds Z_1 to Z_9

Antibacterial Analysis of the Isolated Compounds Z_1 to Z_9

As shown in Fig. 16 and Fig. 18, of the compounds Z_1 to Z_9, only compounds Z_1 and Z_2 from the F_1 fraction had an obvious inhibitory effect on *E. coli* in the antibacterial test. Considering the relatively week inhibitory effect of F_2 (Z_3 to Z_9) on *E. coli* as shown in Table 2, it could be concluded that some bioactive compounds had not been isolated from the F_2 fraction.

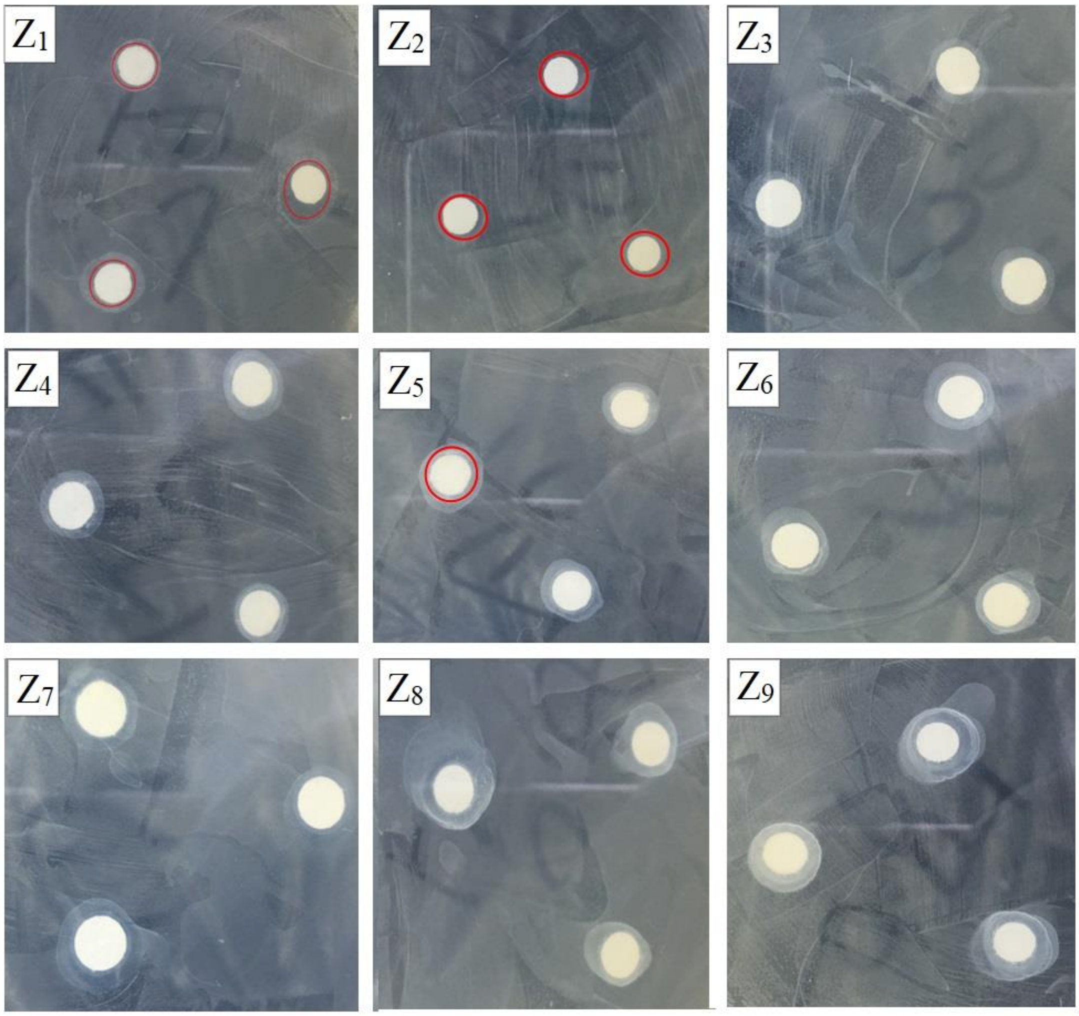

Fig. 16. Antibacterial activity of the compounds Z_1 to Z_9 isolated from the F_1 and F_2 fractions against *E. coli*

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*.

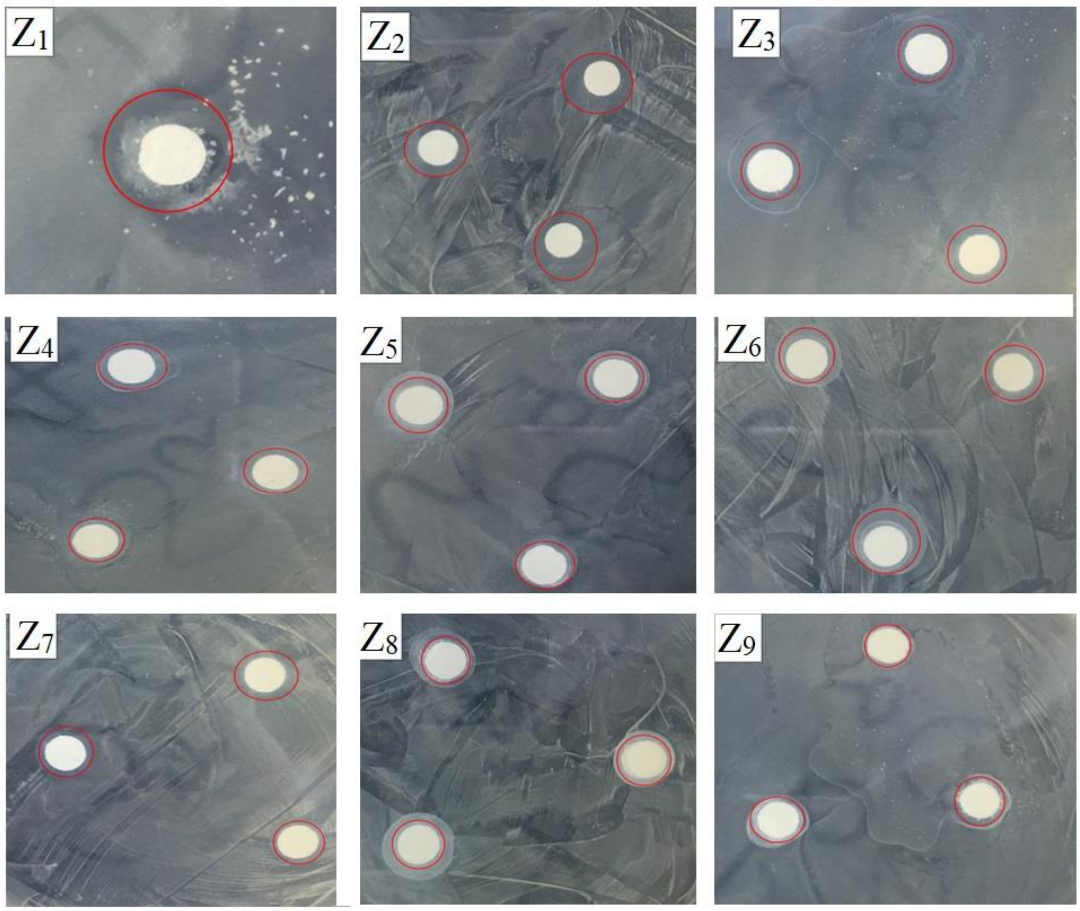

Fig. 17. Antibacterial activities of the compounds Z_1 to Z_9 isolated from the F_1 and F_2 fractions against *S. aureus*

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 Z_1 had a strongest inhibitory effect on *S. aureus* with an average diameter of 12.08 mm. Figures 17 and 18 showed that although Z_2 inhibited the growth of *S. aureus* and produced a clear zone of inhibition, the diameter of the inhibition zone was smaller than that of Z_1. 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 Z_7, Z_8, and Z_9 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 Z_1 to Z_9 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 Z_1 to Z_9 on Gram-positive bacteria was better than on

Gram-negative bacteria. Moreover, it was also found that the compound Z_1 (dehydrodiisoeugenol) had the greatest inhibitory effect on the two test bacteria.

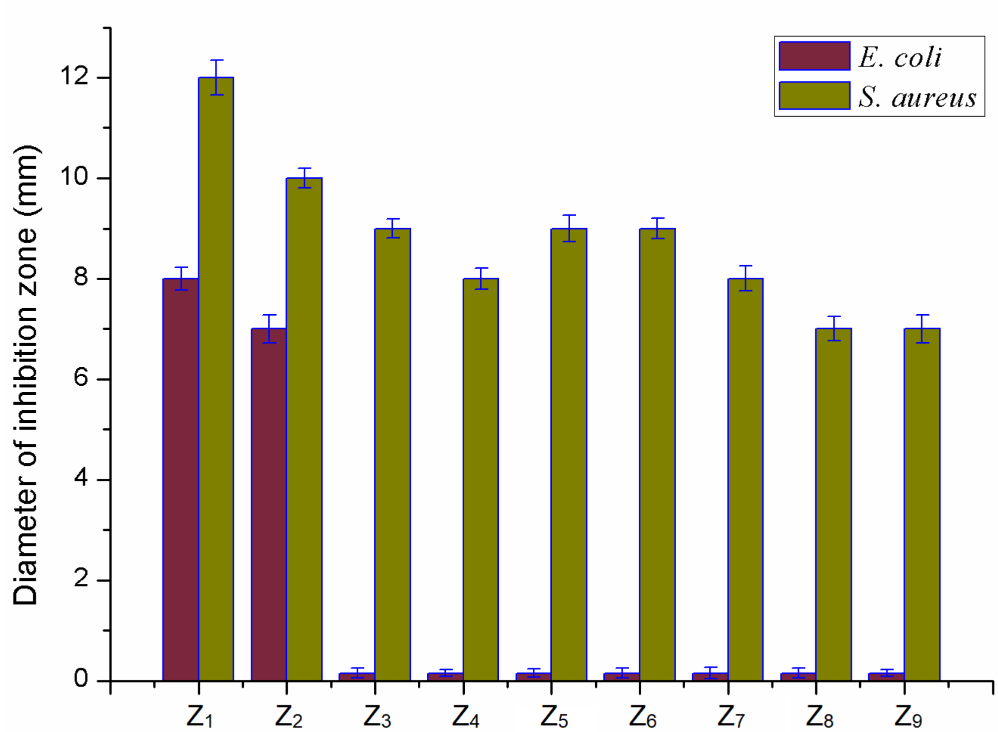

Fig. 18. Zone of inhibition of the compounds Z_1 to Z_9 isolated from the F_1 and F_2 fractions against *E. coli* and *S. aureus*;
Legend: The diameter of inhibition of the samples with no antibacterial activity was subtracted the diameter of the filter paper (6.00 mm)

CONCLUSIONS

1. The four fractions of DHP from isoeugenol, *i.e.*, F_1, F_2, F_3, and F_4 showed remarkable differences in their growth inhibition of *E. coli* and *S. aureus*, with the F_1 and F_2 fractions showing strong antibacterial activity against both species.
2. The bacteriostatic compounds were further separated sequentially by column chromatography from the F_1 and F_2 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 (Z_1-Z_9) 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 (Z_1 and Z_2) could inhibit bacteria such as *S. aureus* and *E. coli*. The Z_3 to Z_9 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.

ACKNOWLEDGMENTS

The authors are grateful for the support of the Nature Science Foundation of China (grant No. 21878070) and the Hubei Provincial Key Laboratory of Green Materials for Light Industry (Q20131402).

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Article submitted: October 27, 2018; Peer review completed: January 1, 2019; Revised version received: January 27, 2019; Accepted: January 29, 2019; Published: February 19, 2019.
DOI: 10.15376/biores.14.2.2842-2860