

# Structural Characterization and Antioxidant Activity of Lignin Extracted from *Ficus Carica* L.

Ibtissem Moussa<sup>1,2</sup>, Ramzi Khiari<sup>1,3,4,\*</sup>, Ali Moussa<sup>2</sup>, Gérard Mortha<sup>4</sup> and Mohamed Farouk Mhenni<sup>1</sup>

<sup>1</sup>University of Monastir, Faculty of Sciences, UR13 ES 63-Research Unity of Applied Chemistry & Environment, Avenue of the environment, 5019 Monastir, Tunisia.

<sup>2</sup>University of Monastir, National Engineering School of Monastir, Street of Ibn El Jazzar, 5000 Monastir, Tunisia.

<sup>3</sup>Higher Institute of Technological Studies of Ksar-Hellal, Department of Textile-Tunisia, Avenue of Hadj Ali Soua. 5070 Ksar-Hellal, Tunisia.

<sup>4</sup>University of Grenoble Alpes, CNRS, Grenoble INP, LGP2, F-38000 Grenoble, France

\*Corresponding Author: Ramzi Khiari. Email: Khiari\_ramzi2000@yahoo.fr.

**Abstract:** The most abundant phenolic biopolymer in the biosphere is the lignin. This phenolic biopolymer commonly exists in combination with polysaccharides and other cell wall components. In this study, the solvent system dioxane-water is used to extract lignin, which is considered as unaltered native lignin. The dioxane lignin extracted from fig stems was characterized regarding to its structural feature, quantification of its functional groups, molecular weight, and evaluation of its thermal properties. Purity and molecular weight distribution of the studied lignin indicated that isolated lignin contained a low amount of sugar (c.a. 19%) and had a high weight-average molecular weight (10 068 g.mol<sup>-1</sup>). Lignin sample had approximately the same amounts of guaiacyl (G) and p-hydroxyphenyl (H) units with relatively fewer syringyl (S) units. The isolated lignin revealed good antioxidant properties. Therefore, it proved to have a high potential of application in new antioxidants formulations.

**Keywords:** *Ficus Carica* L.; lignin organosolv; molecular weight; NMR spectroscopy; antioxidant activity

## 1 Introduction

Lignin is the second most abundant biopolymer on earth containing approximately 15-30% and 12-20% for wood and annual plants, respectively. Lignin presents many advantages that make its utilization quite attractive in a wide variety of applications. These can be recapitulated as (1) various reactive points available for chemical reactions (2) a direct source of several phenolic and aromatic compounds, (3) high energy content because of its aromatic nuclei, (4) promptly accessible in gigantic amounts, (5) if discarded a waste, dark alcohol constitutes a genuine ecological contamination chance and hence another valorization is of interest.

Lignin is typical for vascular plants and particularly abundant in wood. It exerts several physiological key functions [1] such as rendering tissues hard and strong [2], giving them a high resistance to compression (the high weight of the tree itself), and strictly organizing the water transport through the wood structure. The ability to hydrophobic and to strengthen the other cell wall polymers proved to be essential roles for lignin in earthy plants. P-coumaryl, coniferyl, and sinapyl alcohols are the three precursors of lignin. These monolignols originate, p-hydroxyphenyl, guaiacyl, and syringyl phenylpropanoid units, respectively, when incorporated in lignin macromolecule [3]. The type of monomeric units and its relative abundance in lignin structure depend on its botanic origin. In overall, guaiacyl lignins are available in softwoods while contains syringyl and p-hydroxyphenyl structural units can likewise be distinguished. In hardwoods, guaiacyl and syringyl phenylpropanoid units are available in a few proportions together with following measures of p-hydroxyphenyl units. Lignins from annual plants

join guaiacyl and syringyl units at comparable levels, and also extensive amounts of structural elements got from p-coumaryl alcohol. In these lignins, the presence of p-coumaric and ferulic acid residues is additionally frequently observed.

Lignin commonly exists in combination with polysaccharides and other cell wall components [4]. Thus, lignin can occur linked to hemicellulosic sugars such as xylose, arabinose, mannose, and glucose, to cellulose, pectic substances or phenolic acids by ether or ester linkages. Besides, relationship amongst lignins and other cell compounds of polymers, for example, glycol proteins and tannins are noticeable. Extraction of lignin in an undegraded form is mostly considered one of the benefits of the new and many organosolv pulping processes [5]. A various range of 21 pure solvent delignification procedures has been registered by Johansson et al. (1987) [6]. In the standard situation of dioxane extracted lignins, the isolation procedure in dioxane-water (9:1; v/v) with an acid catalyst at different temperatures has been reviewed [7-9]. In the present work, lignin was extracted from FS by a dioxane-water (9:1; v/v) with an acid catalyst. The lignin was characterized taking into consideration its structural features, quantification of its functional groups, molecular weight, and evaluation of its thermal properties. The NMR spectroscopy method was allowed in order to facilitate the investigation into structural aspects of complex lignin polymers. This technique ( $^{13}\text{C}$ -NMR) became the most usual method in lignin analysis. It can be easy for much information such as the determination of the amount of lignin structural. Moreover, the use of this method can estimate the amount of aryl ethers, condensed and uncondensed aromatic as well as aliphatic carbons.

The fig tree is a very widespread tree in Tunisia. In this paper, the identification of dioxane lignin extracted from fig stems (FS) was reported. The content of lignin of FS was 21%. It was vital to complete an entire characterization of the antioxidant potential of dioxane lignin extracted from FS in the light of the fact that the lignin is a significant source of phenolic products [10,11]. Because of the major problems raised by oxidative stress which can have negative impact on human health such as making greater inflammatory or ischemic diseases, cancer, hemochromatosis, etc. It is important to look for a new path, for example, new natural antioxidant products items which can enhance all the detriment related to well-being and particularly for prosperity, particularly for pharmaceutical and cosmetics industries.

## 2 Experimental

### 2.1 Raw Material Preparation

The FS used were obtained from Monastir region (Tunisia) in August 2017. They were washed with distilled water in order to eliminate sand. At that point, they were dried under natural conditions (September 2017). From that point forward, FS was crushed (Wiley mill process), sieved (200-400  $\mu\text{m}$ ) and extracted (Soxhlet mechanical assembly) use a mixture of acetone and dichloromethane. The extractive-free FS test was broiler dried at 40°C (ED115 oven, Binder GmbH, Germany).

### 2.2 Chemicals and Reagents

All the chemical products (Dioxane, hydrochloric acid, 2,2-diphenyl-1-picrylhydrazyl, deuterated dimethyl sulfoxide, pyridine, ethanol, dichloromethane, N,N-dimethyl methanamid, deuterated chloroform, acetone, tetrahydrofuran, cholesterol, chromium(III) acetylacetonate and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) were purchased from Sigma-Aldrich and used as received.

### 2.3 Preparation of Dioxane Lignin from FS

A FS sample (10 g) was treated for 3 h with a mixture of dioxane-water 82:18 (v/v) using hydrochloric acid as a catalyst (0.1 mol.L<sup>-1</sup> HCl in the mixture). After separation of solid and liquid fractions by vacuum filtration, the evaporation of the solvent was done in a rotary evaporator (Heidolph, supply from Germany) at 40°C and 0.9 Mbar which was used to partially remove the solvent. The concentrated residue transferred into three volumes of water in order to precipitate lignin. The precipitate

was recovered by vacuum filtration, washed twice with water and vacuum dried to obtain the dioxane lignin isolated from fig stems (FSdL).

#### **2.4 Acetylation of Lignin**

Acetylation of FSdL sample (600 mg) was conducted using 9 mL of acetic anhydride-pyridine (1:1, v/v) at 25°C for 24 h in a 500 mL flask under vigorous stirring. Then, ethanol (200 mL) was added. After 30 min, the solvents were removed by evaporation under reduced pressure at 45-50°C. Addition and removal of ethanol were repeated more than 10 times to result in a complete removal of acetic acid and pyridine from the sample. The acetylated lignin was recovered and freeze-dried for 12 h. After 48 h, drying the acetylated sample was used for the <sup>13</sup>C-NMR experiment.

#### **2.5 Gel Permeation Chromatography (GPC)**

The molecular weight distribution of FSdL was determined by gel permeation chromatography (GPC) on a PL gel 5 $\mu$  Mixed-D column. The sample was dissolved in tetrahydrofuran (THF) and 200 mL of the sample in the solution was injected. The column operated at 40°C, using THF as mobile phase at a flow rate of 1 mL.min<sup>-1</sup>. Polystyrene standards were used to calibrate the column. The weight-average ( $M_w$ ) and number-average ( $M_n$ ) molecular weights were calculated from their chromatograms. All the results represent the mean of at least triplicate samples. The standard deviations were always observed to be lower than 5%.

#### **2.6 FT-IR Spectroscopy**

The Shimadzu IR prestige-21 spectrometer was used to obtain the FT-IR spectra. 1 mg of lignin and 100 mg of anhydrous KBr, were pressed into small pellets using a laboratory press. The acquisition conditions were 64 scans and resolution of 4 cm<sup>-1</sup>. The wave number scanning was in the range of 400-4000 cm<sup>-1</sup>.

#### **2.7 Quantitative Analysis of <sup>13</sup>C-NMR and <sup>31</sup>P-NMR Spectroscopy**

<sup>13</sup>C-NMR spectra (125 MHz in DMSO) were obtained using a Bruker Avance 300 MHz spectrometer, using deuterated chloroform as a solvent, delay time, 2 second, number of scans: 10240, to characterize the specifications of dioxane lignin. The quantitative analysis of <sup>31</sup>P-NMR was also established using a Bruker Avance 400MHz device equipped with a 5 mm broadband probe. The amount in terms of phenolic, carboxylic and aliphatic hydroxyl groups was determined after derivatization step using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. The test was carried with 30 mg of lyophilized lignin samples which were dissolved in DMF/pyridine (1:1; v/v). Then, they were mixed with pyridine solution containing cholesterol as an internal standard and chromium (III) acetylacetonate as relaxation reagent. For the second test, the derivatization agent was mixed with the lignin solution and CDCl<sub>3</sub> before to move into a NMR tube for spectrum analysis. The elucidation and quantification of the peaks were done according to the literature [12,13].

#### **2.8 Thermal Properties**

Thermogravimetric analyses (TGA) were performed in a Q50 thermogravimetric analyzer, using 7 mg of a sample to determine the mass loss during heating. The sample was heated from 25 to 800°C at a heating rate of 10°C.min<sup>-1</sup> under an oxidizing atmosphere. For TGA analysis, the derivation was performed to obtain the DTG curve. To determine the glass transition temperature, DSC analysis were performed in a Q100 instrument. The lignin sample was analyzed without drying. 5 mg of lignin was placed in a sealed aluminum cell. Nitrogen was used as the vector gas and the following heating program was conducted for the analysis: from 20 to 200°C with a heating rate of 10°C.min<sup>-1</sup>.

## 2.9 Chemical Composition

Acid-insoluble lignin and sugar contents of FSdL were determined after hydrolysis of the sample following the TAPPI T 249 cm-09 standard [14]. Sugars contained in the hydrolysate were identified and quantified by HPLC technique using a Dionex Carbopac PA-1 column (4 × 250 mm) together with a guard column (4 × 50 mm) using a Dionex ICS-3000 system configured for HPLC-PAEC-PAD. Klason lignin content was determined gravimetrically following the standard TAPPI T 222 om-15 [15].

## 2.10 DPPH Radical-Scavenging Activity

Different extract doses (0.3 mL) were mixed with 2.7 mL of a methanolic solution containing DPPH radical ( $6.10^{-5}$  mol.L<sup>-1</sup>). The mixture was shaken energetically and left to remain for 60 min oblivious until the point that steady absorbance esteems were obtained. The absorbance at 517 nm indicated the reduction of the DPPH radical [16]. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using Eq. (1).

$$\% RSA = \frac{(ADPPH - AS)}{ADPPH} * 100 \quad (1)$$

where AS is the absorbance of the solution when the sample extract has been added at a particular level, and ADPPH is the absorbance of the DPPH solution.

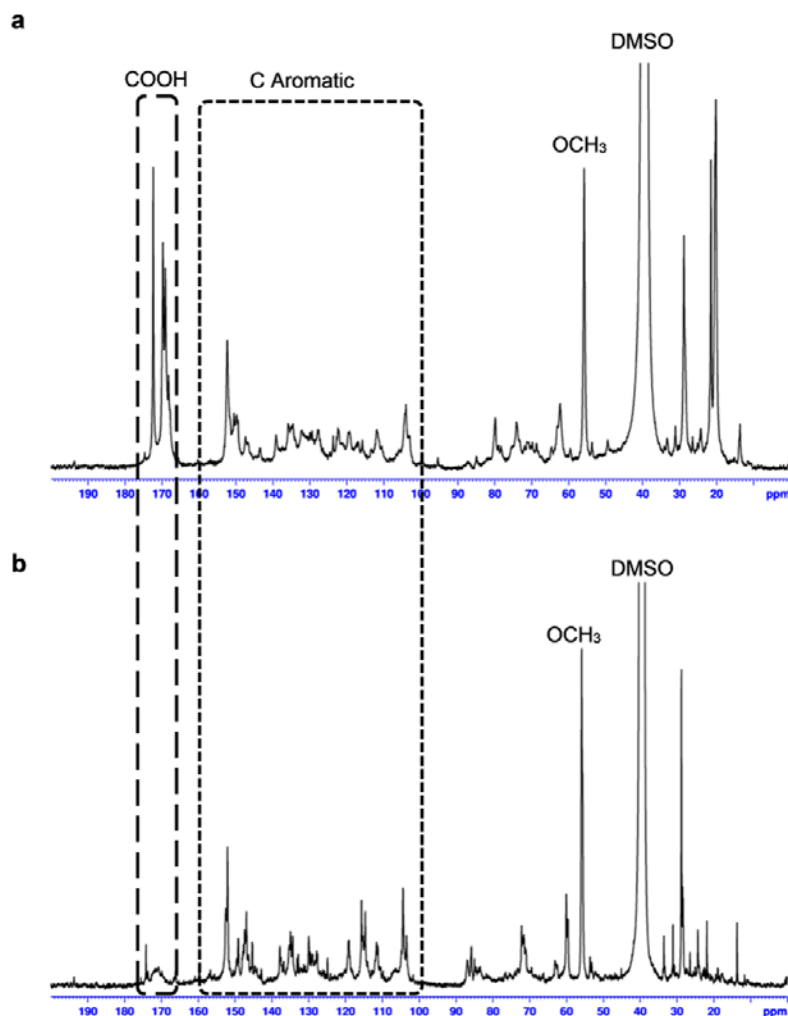
EC<sub>50</sub> is the extract concentration providing 50% of radicals scavenging activity. It was calculated from the graph of RSA percentage against extract concentration.

## 3 Results and Discussion

In this study, after preparation of FSdL, functional groups were identified by FT-IR, <sup>13</sup>C-NMR, <sup>31</sup>P-NMR and GPC techniques. DPPH radical scavenging activity was also evaluated determining antioxidant activity of FSdL to properly propose potential valued applications of this biomass sourced product.

### 3.1 Quantitative <sup>13</sup>C-NMR Techniques

The FSdL and its acetylated derivatives were examined by <sup>13</sup>C-NMR spectroscopy. From the resulting spectra (Fig. 1) some assignments could be made according to the literature [17,18].



**Figure 1:**  $^{13}\text{C}$ -NMR spectra of (a) acetylated and (b) non-acetylated FSdL

Fig. 1 presents the  $^{13}\text{C}$ -NMR spectrum, which demonstrates clearly the disappearance of typical polysaccharide, signals between 60 and 110 ppm. From this figure, it can be noticed that the signal for C-1 (p-coumaric acid ester) was recorded at 125.0 ppm, concerning the aromatic region located between 104 and 165 ppm. Moreover, the syringyl, guaiacyl and p-hydroxyphenyl residues were identified by peaks at 152.3, 138.2, 134.4, 133.3, 132.4, 104.5 ppm (S), 152.3, 149.3, 149.0, 147.2, 145.6, 133.4, 132.4, 119.5, 114.9, 111.5 ppm (G) and 129.8, 127.9, 122.7 ppm (H), respectively. The esterified p-coumaric acid was observed at 167.8, 129.8, 125.0, 115.9 and 115.5 ppm. At 56.0 ppm, the aromatic methoxy group well appears with a large peak. The  $\gamma$ -methyl,  $\alpha$ - and  $\beta$ -methylene groups in n-propyl side chains were observed in the region between 14.0 and 33.8 ppm. The carbonyl resonances, cinnamic acids and esters, acetyl groups and other aliphatic esters, may be identified by a peak at 60.1 ppm. The 4-O-methoxyl group of glucuronic acid residue in the xylan was also observed at 60.1 ppm. This new finding was in a good agreement with the previous study [9] on uranic acids in wheat straw hemicelluloses by  $^{13}\text{C}$ -NMR spectroscopy. In short, the lignin isolated from FS is relatively free of polysaccharide sugars. P-hydroxyphenyl and especially guaiacyl structures are found abundantly in the lignin of FS. These structures are derived from p-coumaryl alcohol and coniferyl alcohol units. In non-woody resources such as bagasse structural units are derived from p-coumaryl alcohol.

Fig. 1(a) shows the  $^{13}\text{C}$ -NMR spectra of acetylated lignin. From this figure, the quantitative  $^{13}\text{C}$ -NMR analysis was used to evaluate the amount of primary, secondary and phenolic hydroxyl groups [19]. The quantity of hydroxyl groups in the lignin was obtained between  $\delta_{\text{C}}$  170-169, 169-168 and 168-167 ppm after shifting the signal between 102 and 160 ppm set as 600. Although numerous studies have assumed more to the  $^{13}\text{C}$ -NMR spectra of lignin, there still remain some problems to attribute for illustration, precise peaks assignments and true quantification based on  $^{13}\text{C}$ -NMR spectra of lignin, which are hard due to signal overlap and other factors.

### 3.2 Quantitative $^{31}\text{P}$ -NMR Spectroscopy

The results of the phenolic group content are given in Tab. 1. FSdL was determined to contain less phenolic hydroxyls than aliphatic hydroxyls (phenolic/aliphatic ratio lower than 1).

**Table 1:** Contents of aliphatic, different phenolic and carboxylic hydroxyl (OH) groups (mmol/g lignin) calculated from  $^{31}\text{P}$ -NMR analysis of FSdL

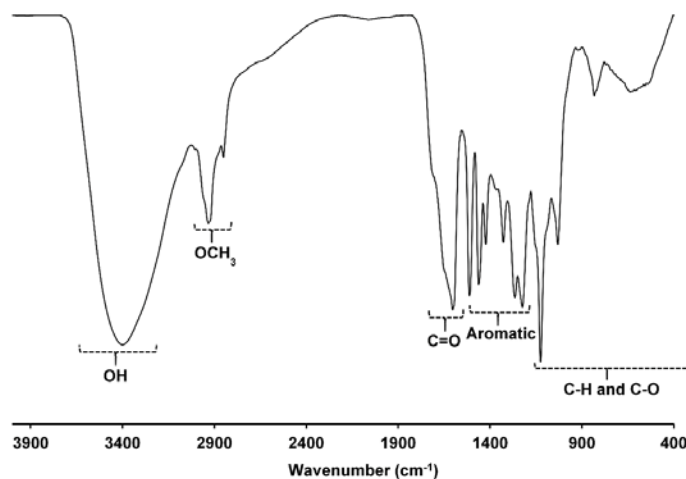
$\text{OH}_S^1$	$\text{OH}_G^2$	$\text{OH}_H^3$	S/G/H ratio	$\text{OH}_{\text{con}}^4$	Cond./uncond (%)	$\text{OH}_{\text{tot al}}$	$\text{OH}_{\text{aliph}}^5$	$\text{OH}/\text{OH}_{\text{ali ph}}$	$\text{OH}_{\text{total}}$	$\text{COO H}$	$\text{OH}_{\text{total}} + \text{COO H}$
0.181	0.465	0.421	17/44/39	0.301	28.210	1.368	3.034	0.451	4.402	0.341	4.743

$^1$ hydroxyl groups in syringyl units;  $^2$ hydroxyl groups in guaiacyl units;  $^3$ hydroxyl groups in p-hydroxyphenyl units;  $^4$  hydroxyl groups in condensed structures;  $^5$  hydroxyl groups in aliphatic structures;

The results also revealed that lignin contained approximately the same amounts of guaiacyl (G) and p-hydroxyphenyl (H) units with relatively fewer syringyl (S) units.

### 3.3 FT-IR Spectroscopy

The result of FT-IR analysis provided information on lignin structure. The spectra contained the characteristic bands absorption of aromatic structures (Fig. 2), confirming the presence of lignin in the sample, O-H stretching band (3400-3500  $\text{cm}^{-1}$ ), C-H stretching band (2850-2950  $\text{cm}^{-1}$ ), and aromatic skeleton stretching band (1600, 1515 and 1430  $\text{cm}^{-1}$ ) [20].



**Figure 2:** FT-IR spectra of FSdL

The FT-IR spectra indicated the presence both of characteristic H and G bands: 1329-1325  $\text{cm}^{-1}$ , 1113-1110  $\text{cm}^{-1}$ , and 832-828  $\text{cm}^{-1}$ . However, the FT-IR analysis would not be enough to confirm the nature of lignin (softwood or hardwood or herbaceous).

### 3.4 Purity of Lignin Sample and Molecular Weight Distribution

The purity of dioxane lignin was established as insoluble (Klason lignin) (Tab. 2). Dioxane lignin was extracted from the FS and the value was c.a 72%.

**Table 2:** Effect of sugar content on the weight-average molecular weight ( $M_w$ ), number-average molecular weight ( $M_n$ ), number-average degree of polymerization ( $DP_n$ ), weight-average degree of polymerization ( $DP_w$ ) and the polydispersity index ( $D = M_w/M_n$ ) of FSdL

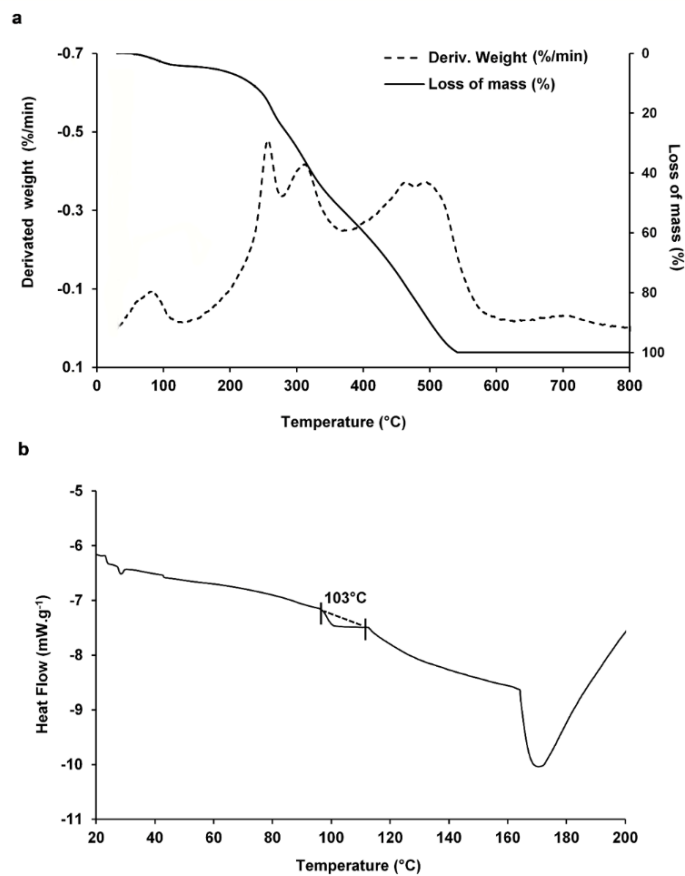
Klason lignin (%)	Sugar (%)	Relative sugar composition (%)		$M_n$ ( $\text{g.mol}^{-1}$ )	$DP_n$	$M_w$ ( $\text{g.mol}^{-1}$ )	$DP_w$	$M_w/M_n$
		Glucose	Xylose					
72.43	18.98	38.90	61.1	5 424	52	10 068	97	1.86

The FT-IR spectra (Fig. 2) of lignin led to the same deduction: a high purity of lignin sample as demonstrated by the FT-IR spectra: absence of peaks related to the inorganic compounds (absence of absorption bands around 600  $\text{cm}^{-1}$ ). Xylose was the most important sugar for FSdL, which is consistent with the nature of the major softwood and hardwood hemicelluloses. The weight-average molecular weight ( $M_w$ ), number-average molecular weight ( $M_n$ ), number-average degree of polymerization ( $DP_n$ ), weight-average degree of polymerization ( $DP_w$ ) and polydispersity index ( $D = M_w/M_n$ ) are given in Tab. 2. The relative sugar analysis showed a perfect trend to xylose and glucose residues with high molar mass and low polydispersity.

### 3.5 Thermal Properties

The results of thermogravimetric analysis, shown in Fig. 3(a), demonstrate that the temperature at which the lignin begin to degrade is situated around 180°C.

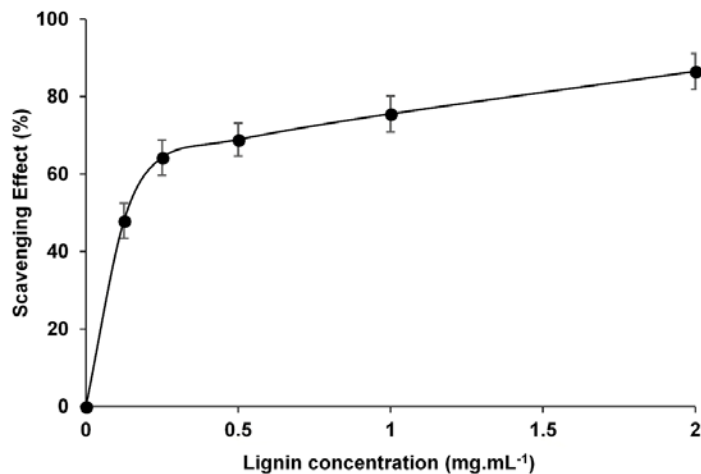
The temperatures of maximal degradation rates were obtained from the first derivation of the TGA curves. It could be seen from figure that the temperature of the maximum degradation rate for the lignin was 314°C. For a mass loss of 50%, the temperature was 360°C. Fig. 3(a) shows that lignin degradation is taking place within a large range of temperature (180-550°C) under air, which could be attributed to the complexity of the lignin structure. Indeed, this vast range of degradation temperatures was explained by various thermal stabilities of different functional groups containing oxygen. In fact, during the thermal degradation of extremely cross-linked lignin polymer, the cleavage of oxygen functional groups followed by a total rearrangement could take place. It would lead to the creation of a complex structure more stable at a higher temperature. The glass transition temperatures ( $T_g$ ) were determined by DSC (Fig. 3(b)). The  $T_g$  value obtained for FSdL was 103°C.



**Figure 3:** (a) TGA and DTG curves, (b) DSC curve of FSdL

### 3.6 Antioxidant Activity

Fig. 4 illustrates the results of the radical scavenging activity (RSA) values, which were assessed as the ration percentage of absorbance decrease, and the observance of DPPH solution in the absence of extract measured at 517 nm.



**Figure 4:** Scavenging effect of FSdL



From this figure, the RSA impacts of lignin against DPPH radicals showed remarkable marks, which were justified by the obtained, trend behavior. The antioxidant activity  $EC_{50}$  ( $\mu\text{g}\cdot\text{mL}^{-1}$ ) value of FSdL was determinate (i.e.,  $192.459 \pm 1.634$ ). The  $EC_{50}$  value obtained for this extract was excellent (less than  $200 \mu\text{g}\cdot\text{mL}^{-1}$ , average value). FSdL revealed very good antioxidant activity.

The obtained result is generally in accordance with the total phenol and phenol condensed contents shown in Tab. 1. As reported by Barreira et al. [21] a negative linear correlation between  $EC_{50}$  antioxidant activity values and the total phenols content can be detected. This can be noticed also during the work by Velioglu et al. [22] that the samples with highest total phenols amount exhibit lower  $EC_{50}$  amount, confirming that phenols are likely to contribute to the antioxidant activity of the extracts. The result of the phenol condensed was also correlated with the result of  $EC_{50}$  scavenging capacity value with comparable correlation coefficient value. In conclusion, the dioxane lignin isolated revealed good antioxidant properties, with low  $EC_{50}$  value.

#### 4 Conclusions

The results obtained from GPC, FT-IR, and NMR analysis are in good agreement regarding the less structural changes and degradations of FSdL. GPC analysis show high molecular weight and low polydispersity index for the FSdL. It can be concluded that less degradation of lignin has taken place. Lignins are considered low-value residues, but the utilization of green methods for their recovery and with the results available from their structural analysis, the sustainable application of technical lignins will become more practical. Further researches would be needed to determine the best applications of FS lignin. They may also find applications (antioxidant, UV stabilizer or fire-retardant).

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