

Thermal, Morphological and Cytotoxicity Characterization of Hardwood Lignins Isolated by *In-Situ* Sodium Hydroxide-Sodium Bisulfate Method

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Abstract

In the present work, lignin is isolated from three different agro-industrial waste, sweet sorghum, rice straw and sugarcane bagasse using *in-situ* sodium hydroxide-sodium bisulfate methodology. Characterization was performed using fourier transform infrared analysis (FTIR), scan electron microscopy (SEM), thermo gravimetric analysis (TGA). The SEM micrographs showed sponge-like structure except for sugarcane bagasse lignin reveals rock-like structure. The FTIR indicates the presence of hydroxyl, carbonyl and methoxyl groups in the lignin structure. TGA thermograms were relatively same and sugarcane bagasse lignin was found the most thermally stable up to 201°C as compared to both of soda and kraft sugarcane bagasse lignin and its maximal temperature degradation rate DTG_{max} was found at 494°C while 450°C, 464°C in addition to thermal stabilities up to 173°C and 180°C for sweet sorghum and rice straw lignins respectively. All lignins exhibited low percentage of bio-char less than 10% remained unvalotilized at the end of the thermogravimetric analysis at 800°C in nitrogen atmosphere, revealing a high conversion yield into volatiles. Moreover, all lignin samples depicted higher cytotoxic potential towards lung cancer cell line (A549), IC_{50} : 12 - 17 µg/ml. These findings suggest that the *in-situ* separated lignins would be good candidates for pyrolysis, polymer composites preparations and seem to be promising natural anti-cancer agents despite its main utilization as the caner drug delivery substrates.

Keywords

Lignocellulose, Lignin, Sweet Sorghum, Rice Straw, Sugarcane Bagasse, FTIR, SEM, TGA, Cytotoxicity

1. Introduction

Dwindling fossil fuel resources as well as global climate change due to green house gas emission (GHG) [1] is a global concern nowadays and makes fuel based on the exploitation of biomass, an excellent alternative for both environment and development of bio-economy [2] [3] [4]. Lignocellulosic biomass ranges from forestry to agricultural residues are an abundant and a renewable resource on earth and can be converted into value-added products [5] by the main three steps, biomass pre-hydrolysis treatment, hydrolysis and fermentation.

Among the different lignocellulosic biomasses are rice straw, sweet sorghum and sugarcane bagasse comprises (25% - 50% cellulose, 15% - 30% hemicelluloses and 15% - 25% lignin and minor other extractives) which are produced extensively in Egypt during grain and sweet sugar production as an agro-industrial waste, considered a potential for cellulose and lignin based industries [6]. These constituents could be used for the production of cellulose derivatives, plastics or could be used potentially as a substrate for the release of monomeric sugars essential for second-generation biofuel production, also they were used traditionally for a firewood, building materials, animal food, pulp industry and recently biosorbents [7].

Lignin is an underexploited side-stream low value material produced from paper industry and biorefineries. Its properties mainly depend on its native source and the isolation process.

Lignin is typically found in soft wood with a range of 25 - 35 wt% of dry matter, whereas the lignin content in hard wood is slightly lower 18 - 25 wt% [8], it is a highly present aromatic polymer on earth and the second frequently present organic polymer after cellulose and it is by far the most important source of aromatic compounds for the chemical industry [9] as well as pharmaceutical applications [10] [11] [12]. Out of the three main polymers in plant, lignin has the most complex, hydrophobic and heterogeneous in composition and structure. This tridimensional amorphous biopolymer is built up from phenyl propanoid (phenylpropane) units which are substituted at various positions, linked by ether and C-C bonds. There are three basic building block structures abbreviated H (p-hydroxyphenyl), G (guaiacyl) and S (syringyl), differing in the number of methoxy groups on the aromatic ring; 0, 1 or 2, respectively. These structures can be represented in their alcohol form; namely as p-coumaryl, coniferyl, and sinapyl alcohols, **Figure 1**, these primary lignin building blocks originate from the shikimate pathway [13]. Lignin plays a key role in construction and strength to plant cell walls, changeable fluid movement to protect plant cell wall from microbial invasion [14].

Lignin is recognized as a highly branched amorphous biopolymer with a variety of functional groups including hydroxyl (aliphatic and aromatic), carboxylic, carbonyl and methoxyl groups [15] and can be classified into two main categories, Sulfur-free lignin and sulfur-containing lignin [16] according to the separation process.

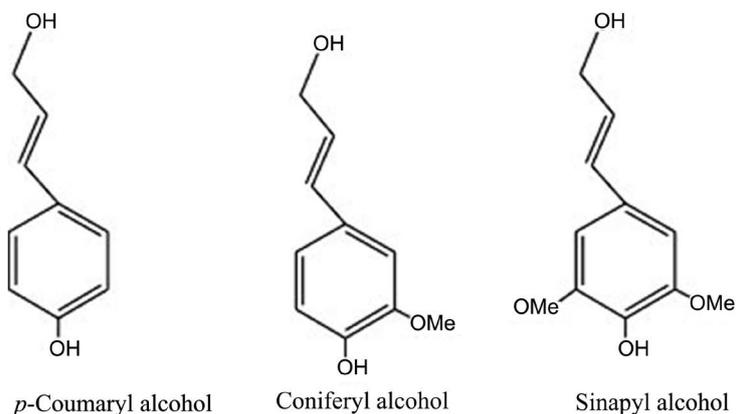


Figure 1. The building blocks of lignin structure.

Currently there is a deep interest in biomass pretreatment technologies, which is the key factor in the second generation biorefinery commercialization and to obtain useful materials and products related to all biomass constituents enhance the economic viability of these lignocellulosic biorefinery [17] [18] [19] where lignin could be privileged into high-value added materials.

The lignin structure usually undergoes chemical modification during biomass pretreatment alter its native structure [20] and this drawbacks and restricts many of its potential industrial applications [20], therefore a successful biomass pretreatment technology must avoid both of lignin fragmentation, condensation reactions and demethoxylation.

One of the most common methods used to recover biomass lignin is steam explosion of fiber followed by a mild alkali extraction which is well known for fractionalizing lignocellulosic biomass into its main components [21] [22] [23], in addition to, there are four main technical lignins produced from pulping which are kraft lignin, soda lignin, lignosulfonate and organosolv lignin [24] relatively maintains its native structure.

The main objective of this study to follow up the morphological features change and thermal properties as well as cyto-toxicity of sweet sorghum, rice straw and sugarcane bagasse lignin recovered by a recently described *in-situ* sodium hydroxide-sodium bisulfate method using FTIR, SEM and thermal analyses to investigate its effect on the structural changes of these lignins which are categorized as a sulfur-free lignin that may evolve into industrial-scale value-added products [25] [26]. The *in-situ* process depends on treatment of lignocellulosic biomass using endothermic sodium hydroxide step followed by exothermic sodium bisulfate step generated *in-situ* in 42.2% yield. This method shows hemicelluloses solubilization 89%, lignin removal 91%, cellulose loss 5.8% and acellulosic substrate composition 87.6% cellulose, 4.9% hemicelluloses and 5.7% lignin as well as a negligible detection of furfural and 5-hydroxymethyl furfural [27] [28].

2. Experimental

Materials and methods:

Rice straw, sweet sorghum and sugarcane bagasse were provided from local Egyptian farmers in exception of the later provided from Abo-Korkas sugar mill factory, Elminia Governorate, Egypt. Chipped into small pieces 1 cm length and oven dried at 105°C for 48 h. before conducting pretreatment experiments.

In situ sodium hydroxide-sodium bisulfate pretreatment:

The pretreatment was conducted using autoclave systec VE-75 using the described procedures. treatment of lignocellulosic biomass using sodium hydroxide 1.3% at 120°C for 40 min. then treatment using sodium bisulfate generated in situ in an exo-thermic step, 42.2% yield, by adding equimolar amount of sulfuric acid to the slurry and keeping the temperature at 105°C for further 40 min, total reducing sugar-containing hydrolyzate was determined using previously described procedures [29]. The resulting compressed wet substrate is then post-treated with 0.2% - 1% sodium hydroxide stirring on cold for lignin recovery and used for characterization without any further purification [27] [28].

Fourier Transform Infra-Red Analysis:

FTIR spectra were recorded on Nicolet 6700 spectrophotometer using potassium bromide.

Scan electron microscopy:

Lignin samples were dried and coated with gold and were imaged using scan electron microscopy, JOEL-JSM 5400 LV (Japan).

Thermogravimetric Analysis:

The study of the thermal properties was carried out by means of a thermogravimetric analysis (TGA) and first derivative of this curve (DTG), using a Shimadzu thermal analyzer DTG 80-H (Japan). Scans were run from 30°C to 800°C with a heating rate of 10°C/min under a flow of nitrogen of 40 ml/min.

Cytotoxic activity:

Cytotoxic effect of the three lignin samples was tested on lung cancer cell line A549 by using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT Assay). A549 lung cancer cells at a concentration of 10⁵ cells/ml were inoculated into 96 well tissue culture plate and incubation was maintained at 37°C under conditions of 5% and 95% of air for 24 h to develop a monolayer sheet before the treatment of the lignin compounds which after then were dissolved in DMSO and diluted in RPMI medium with 1% serum. Cells were treated with four different concentrations from each lignin sample for the next 24 h. MTT solution was prepared at concentration of 5 mg/ml and then 20 µl MTT solution was added to each well and incubated the plate for 4 - 5 h. 200 µl DMSO was used to dissolve the insoluble formazan and the purple optical density was measured at 570 nm which is directly proportional to the cell viability [30].

3. Results and Discussion

For lignin composition, lignin samples were characterised by means of the determination of its content in Klason lignin and ashes. Ash content was determined gravimetrically, after combustion of the sample at 800°C ± 25°C for 3

hours in a muffle furnace [31]. Klason lignin content was also determined gravimetrically after acid hydrolysis of the sample [32], for sugar analysis, Aliquots of 3 ml of 72% H₂SO₄ were added to 1 g samples of lignin in pressure tubes. The tubes were placed in a water bath at 30°C for 1 h and stirred intermittently to completely wet the lignin sample. The solution was then diluted to 50 ml through the addition of water and the samples were autoclaved in pressure tubes at 121°C for 1 h. The samples were further diluted to 100 ml, filtered with porcelain crucibles to remove solids and the liquid fraction was neutralized using sodium bicarbonate and analyzed for total reducing sugar using the mentioned procedures [29], **Table 1**.

FT-IR Analysis:

The effects caused by the different lignin polymeric structures in the FTIR spectra are presented. The recorded spectra of lignin samples were compared with its assignments.

All lignins showed a strong wide band within the range 3400 and 3100 cm⁻¹, assigned to the hydroxyl groups in both of phenolic and aliphatic structures. The two bands located at 2919 and 2850 cm⁻¹, due to the symmetrical and asymmetrical CH stretching of the methyl and methylene groups are also noticed in the three samples.

The absorption band of 1649 cm⁻¹ was identified in all three lignin samples is assigned to the stretching vibrations of conjugated carbonyl groups, there are some bands which are present in all the studied lignin fractions, such as bands located at 1595 and 1510 cm⁻¹, related to vibrations of aromatic rings, and two other bands attributed to methoxyl groups present at 1455 and 1424 cm⁻¹.

Thermal analysis:

The thermogravimetric (TG) analysis indicates weight loss of lignin samples in relation to the temperature of degradation, and the first derivative of that curve (DTG) which shows the corresponding rate of weight loss for every one of the obtained samples, respectively. The peak of this curve (DTG_{max}) may be expressed as the temperature of maximal degradation rate.

Sugarcane bagasse lignin, **Figure 2**, showed the highest value of DTG_{max} (494.76°C), and its thermal stability around 201°C while for sweet sorghum and rice straw lignins, **Figure 3** and **Figure 4** respectively, the obtained values of DTG_{max} were 450.66°C and 464.33°C, and their thermal stabilities around 173°C and 180°C respectively. Thus, sugarcane bagasse lignin is the most thermally stable sample of this study and also as compared to sugarcane bagasse lignin isolated

Table 1. Klason, ash and total sugar content in lignin samples separated by *in-situ* method.

	Klason lignin%	Ash%	Total reducing sugars%
Sweet sorghum lignin	92.41 ± 0.55	0.98 ± 0.08	4.14 ± 0.13
Rice straw lignin	91.89 ± 0.49	1.12 ± 0.08	4.14 ± 0.16
Sugarcane bagasse lignin	90.87 ± 0.61	0.96 ± 0.09	5.06 ± 0.15

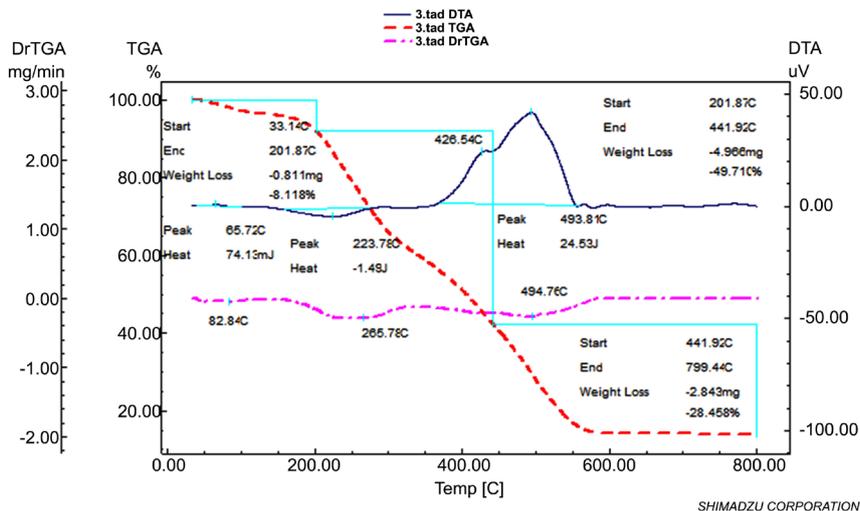


Figure 2. Thermal analysis of sugarcane bagasse lignin.

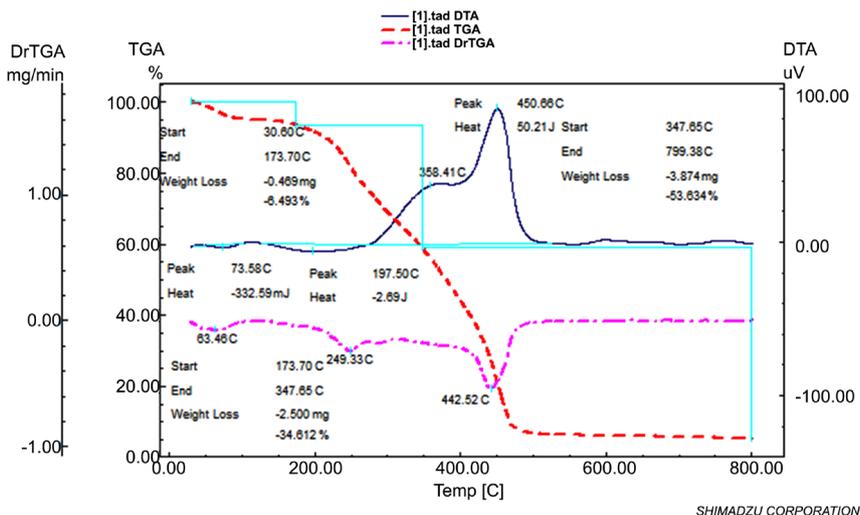


Figure 3. Thermal analysis of sweet sorghum lignin.

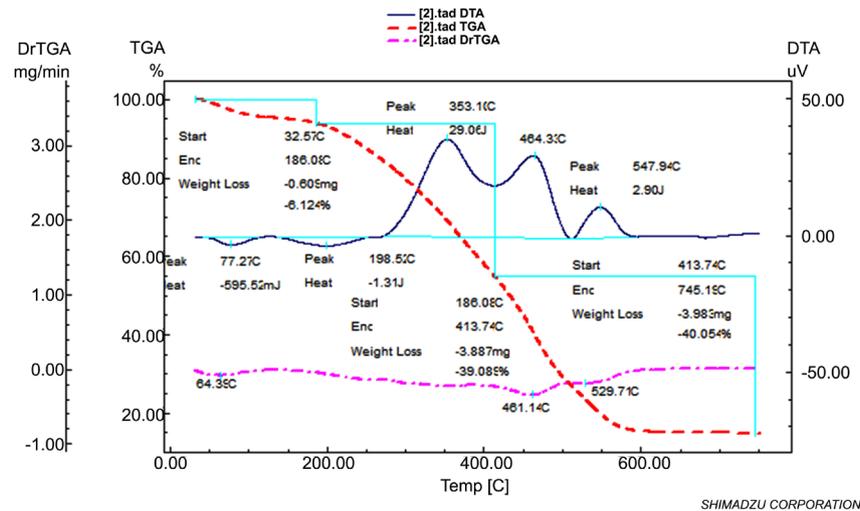


Figure 4. Thermal analysis of rice straw lignin.

by the soda process which is thermally stable around 175°C and its maximal degradation temperature was 380°C [33], and also as compared to kraft sugarcane lignin which is thermally stable below 190°C and its maximal degradation temperature was 377°C [34].

At the end of the thermogravimetric analysis (800°C, in a nitrogen atmosphere) a very low percentage of residues remain unvolatilized, bio-char and/or highly condensed aromatic compound is obtained, in contrary to many literature [35] regarding thermal degradation of different lignin resources, also disagrees to both of soda sugarcane bagasse lignin [33] and sugarcane bagasse kraft lignin [34] that gives a high percentage of these unvalotalized residues at the end of thermogravimetric analysis strongly indicates the *in-situ* separated lignins are higher in methoxy content [36] giving a very low unvalotalized residues and consequently a good candidate for pyrolysis.

Degradation of the lignin samples can be divided into three stages [37] [38]. In stage one, the initial weight loss step occurred at 30°C - 120°C due to the evaporation of water absorbed. Stage two is seen to take place around 180°C - 350°C and is attributed to the degradation of components of carbohydrates in the lignin samples, which are converted to volatile gases such as CO, CO₂, and CH₄. The final stage of degradation occurred over a wide range of temperatures above 350°C. Within this stage, degraded volatile products derived from lignin including phenolics, alcohols, aldehyde and acids along with the formation of gaseous products are getting removed.

Scan Electron Microscopy, SEM micrograms: (Figures 5-7)

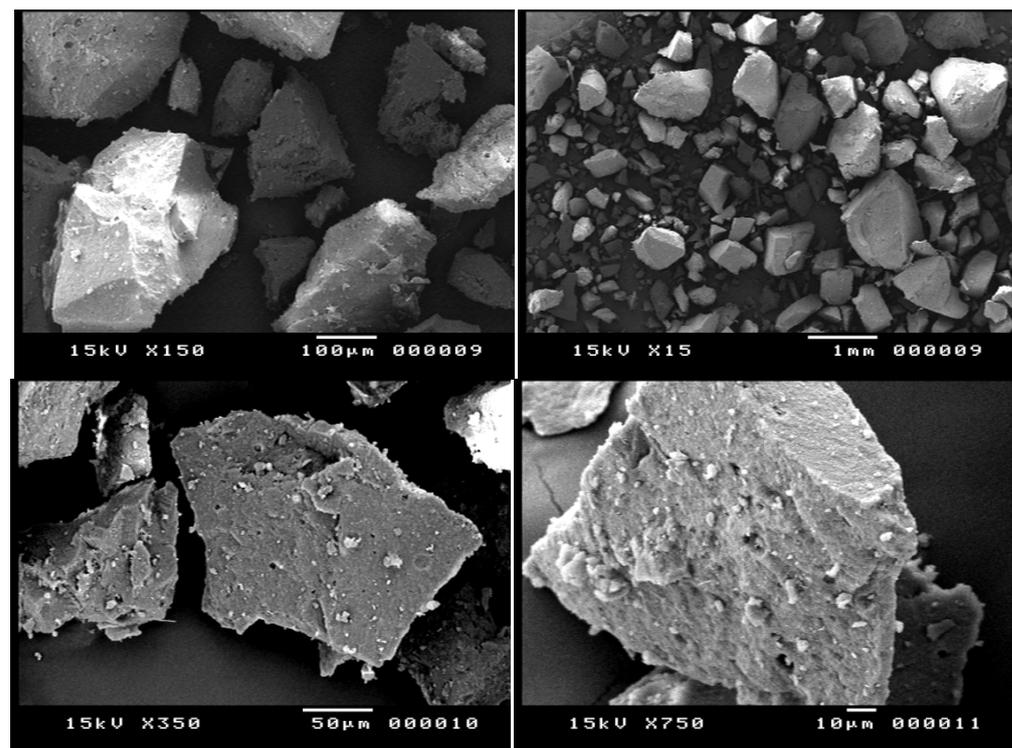


Figure 5. SEM micrographs of *in-situ* sugarcane bagasse lignin.

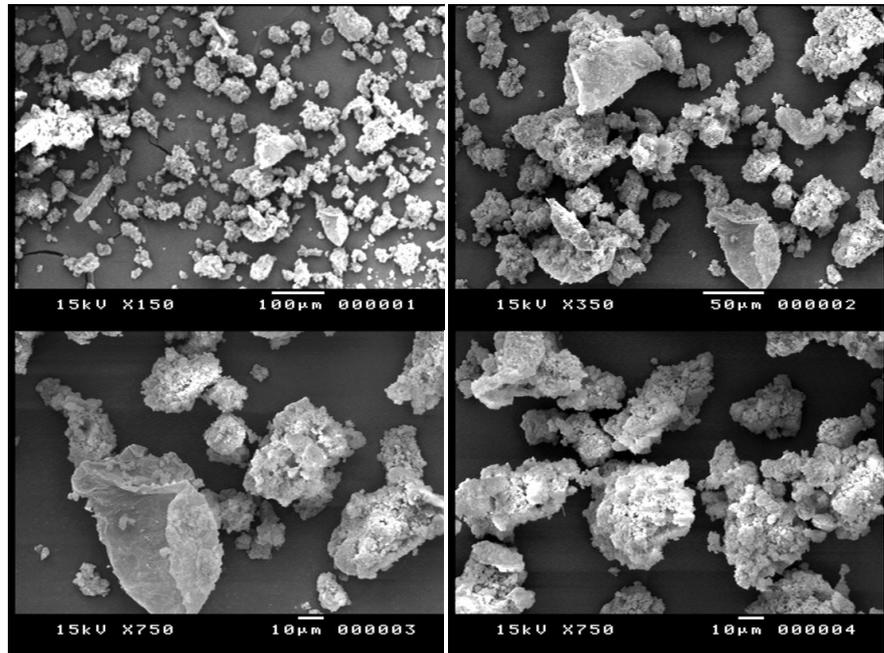


Figure 6. SEM micrographs of *in-situ* sweet sorghum lignin.

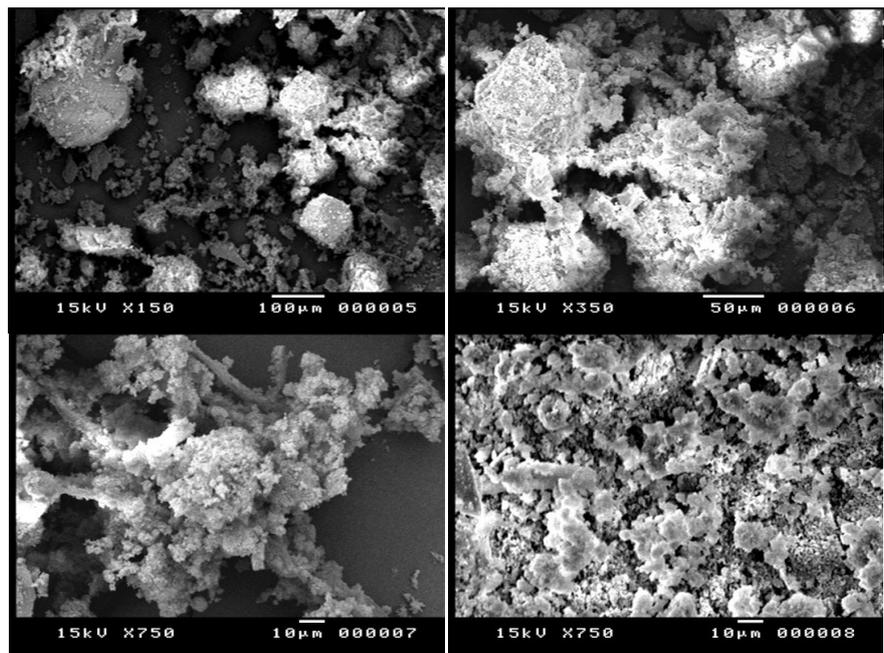


Figure 7. SEM micrographs of *in-situ* rice straw lignin.

The scan electron microscope morphologies of both of sweet sorghum and rice straw lignin reveal sponge-like structures with a high degree of agglomeration in case of rice straw lignin, in contrary to sugarcane bagasse lignin which obviously appears as rock-like structure with different particle size and relatively porous surface.

Cytotoxicity activity:

All the three lignin samples separated by the *in-situ* method had a remarkable

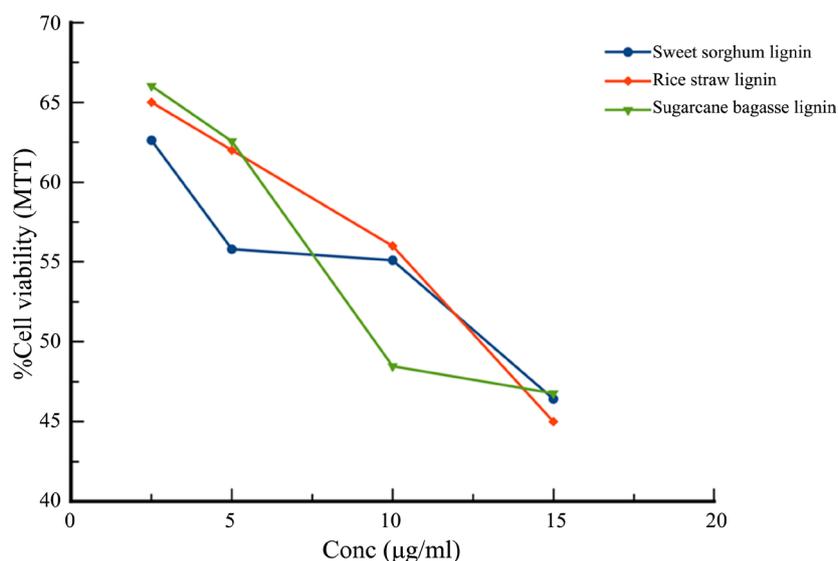


Figure 8. Cytotoxic effect of sweet sorghum, rice straw and sugarcane bagasse lignin towards lung cancer cell line A549, at different concentrations and conducted in triplicate.

Table 2. The corresponding IC_{50} value for each lignin.

Compound	IC_{50} µg/ml
Sweet sorghum lignin	12.5
Rice straw lignin	17.9
Sugarcane bagasse lignin	12.2

cytotoxic effect towards lung cancer cell line A549 giving IC_{50} value, a concentration of a sample that is required for 50% inhibition *in-vitro*, ranges 12 - 18 µg/ml suggest the potential of the three lignin samples as a natural anti-cancer agents and the results show sugarcane bagasse lignin is the most promising despite the fact it has well been used as a cancer drugs delivery substrates due to its non toxicity and good physiochemical compatibility as well as bio-degradable properties (Figure 8 & Table 2).

4. Conclusion

Three types of lignocellulosic biomass lignin samples were isolated using *in-situ* sodium hydroxide-sodium bisulfate method and characterization was performed using fourier transform infrared spectroscopy, thermogravimetric analysis and Scan electron microscopy as well as cytotoxicity effect was examined. The results reveal the *in-situ* lignin samples were thermally stable in addition to low bio-char formation at high temperatures under nitrogen atmosphere indicated its suitability in polymer composites preparation and good candidates for pyrolysis and the cytotoxicity results suggest these lignin samples would be a natural anti-cancer lead.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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