# Antioxidant, antibacterial and antitumoural activities of kraft lignin from hardwood fractionated by acid precipitation

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## Abstract

Kraft lignin, so far useful for energy generation, has been gathering considerable attention as an alternative material to replace fossil-based resources mainly due to its high phenolic content. However, the wide molecular weight distribution and chemical composition heterogeneity led to the development of fractionation methods. Herein, to narrow such characteristics we used eucalypt kraft lignin fractionated at pH's 9, 7, 5 and 3 by sequential acid precipitation. These lignin fractions were first characterised by simultaneous pyrolysis and trimethylsilylation (SPyT) with N-Methyl-N-(trimethylsilyl) trifluoroacetamide with posterior tests of antioxidant, antibacterial, and antitumour activities. We observed higher ratio of syringyl/guaiacyl groups and increase in antioxidant activity in those fractions with lower molecular weight (precipitated at lower pH's). Fractions precipitated at pH's 9 and 7 have shown an outstanding antibacterial activity against five bacteria. Moreover, fractions 7 and 5 presented at cytotoxicity tests ability to inhibit the growth of U87MG and T98G glioma cells, while only a slight inhibition of adult human fibroblasts (non-tumour cells) was detected.

*keywords*: $IC_{50}$; technical lignin; radical scavenging; antibacterial; antitumoural; hardwood lignin

## 1 Introduction

Lignin is a natural renewable phenolic polymer built up of phenylpropane structures, which depending on the methoxylation degree is called *p*-hydroxybenzyl, guaiacyl or syringyl [1]. In plant, lignin protects against biochemical stress, and is responsible for rigidity, bringing strength and avoiding water cell elements to collapse [2]. After isolation, this main source of aromatic compounds is considered a potential precursor to produce high value-added bio-based products [3]. It was reported that lignin does not elicit damage to cells, and it is also biodegradable, environment-friendly, biocompatible and has been proposed as a versatile material applied in biomaterials and food packaging [4]. Furthermore, it is also reported to be anticancer, anti-hypercholesterolemia, antiviral, antimicrobial and antioxidant [5,6]. However, this complex and amorphous material can have its structure and properties severely modified during the biomass pre-treatment [7]. Therefore, the potential outcome is highly restricted by its features.

The main contributors for an effective antioxidant from lignin comes from the phenolic hydroxyl groups, methoxy, aliphatic hydroxyl and double bond between the outermost carbon in the side chain [4]. The phenolic hydroxyl group has been extensively reported as the main structural descriptor for the antioxidant activity of polyphenols, due to the hydrogen atom ability to scavenge free radicals [8]. Moreover, increase in molecular weight, polydispersity and heterogeneity can decrease its antioxidant capabilities. In addition, the antimicrobial effect of lignin can be related to the aromatic compounds, which functions similar to antibiotics such as methicillin [5]. Furthermore, the recent antitumoural activity of lignin reported in the literature includes non-human cells. A variety of tumour models such as ovary, breast and skin tumours were investigated from mouse, monkey and hamster cells [9–11].

Besides the interesting structure of lignin, the large availability of technical lignins and its non-food character are other factors leveraging efforts on lignin valorisation. Currently, the produced lignin is mostly used as internal power supply and less than 2% is recovered for utilization as a chemical product [5, 11]. The kraft lignins present non-uniform chemical structures [13] and wide molar mass distribution [14], which affect its further solubility and reactivity. To overcome such heterogeneity, and for fragmentation and/or purification processes, to obtain specific lignin fractions, became challenging but mandatory [15].

Therefore, approaches were investigated to obtain specific lignin fractions from black liquor in order to obtain a specific molecular weight distribution and tuneable compounds. The most common ones consist of organic solvent extraction [16], selective acid precipitation [17,18] and ultrafiltration technique [19,20]. From these methods, selective acid precipitation is a

simple and economically viable approach. Our group previously isolated hardwood kraft lignin from black liquor by a sequential acidification process, and the effects of acid precipitation on the properties of generated fractions were investigated [21,22]; the pH-fractionated hardwood kraft lignin differed in molecular weights, chemical composition and yields.

Although the lignin bioactivity has been reported, the gap between the isolated lignin characteristics and the heterogeneity of isolated lignin itself, lead to a different structure-property correlation. Therefore, we fractionated kraft lignin, narrowing its heterogeneity and generating fractions from the same source however with differences in molecular weight and chemical composition. Different from what has been reported so far [9–11], our *in vitro* antitumoural tests were performed with human cell models, specifically with two gliomas (grade IV tumours derived from central nervous system) and a cell line of adult human fibroblasts (non-tumour cells) for comparison. The gliomas, U87MG and T98G, are two lines well described in the literature, which present responsive and resistant responses, respectively, to the conventional treatment with temozolomide - TMZ. By doing so, it was possible to observe the different lignin fractions behaviour at similar tumour model -derived from human cells. Our approach enabled a more straightforward correlation between lignin characteristics and its antioxidant and bioactivity properties (Figure 1).

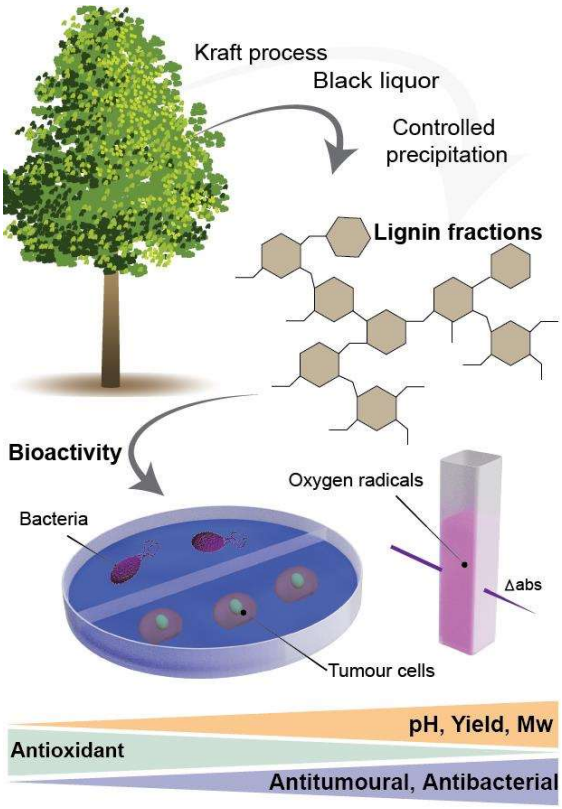


**Figure 1**. Overall scheme on lignin fractionation and its antioxidant and antimicrobial activities.

## 2 Materials and Methods

### 2.1 Isolation of lignin

The lignin used in this study refers to fractions precipitated from eucalypt kraft black liquor provided by Suzano Pulp and Paper Industry. The fractionation method is thoroughly described in a previous work [21]. Briefly, diluted hydrochloric acid (started with a 12 M concentration followed by a 1 M) was slowly added to the black liquor until reached pH 9, then it was centrifuged at 3500 rpm for 15 min resulting in a supernatant and a precipitate. The supernatant was acidified using HCl at the same concentration and technique for black liquor and centrifuged again to get next supernatant and precipitate parts. This procedure was repeated to obtain samples at pH 7, 5 and 3. The precipitated materials in each of these pH's were suspended at the same pH and centrifuged; afterwards, the solid was washed with acidified water (*ca.* pH 2), oven-dried (50ºC) and milled in a mortar. The yield (%) recovered in each fraction was 50 ± 0.1 (fraction 9), 38 ± 2.3 (fraction 7), 7.5 ± 2.6 (fraction 5) and 5.1 ± 0.9 (fraction 3).

### 2.2 Characterization of lignin fractions

The determination of carbohydrate and lignin contents in the lignin fractions is previously described in Lourençon et al., 2020 [22], and the average molecular weight (Mw), determined by gel permeation chromatography, is described in Lourençon et al. (2015) [21].

Simultaneous pyrolysis and trimethylsilylation (SPyT) with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were used for molecular characterization. Pyrolysis was carried out in a heated-filament pyrolyser with a platinum coil and quartz sample tubes. The solid materials (ca. 750 µg) were on-line silylated with 15 µL of MSTFA with 5% (by vol.) pyridine. SPyT was performed at 550 ºC for 20s using CDS 5000 pyroprobe, with the coil filament heater set at 15 ºC $ms^{-1}$. The accessory temperature program: 50 ºC to 280 ºC (100 ºC $min^{-1}$) held for 120 s; the transfer line and oven were also heated to 280 ºC. SPyT products were analysed by gas chromatography - mass spectrometry (GC-MS). A 5 mm diameter split/splitless injector was used (230 ºC) with a split ratio of 1:100. Trimethylsilyl-derived compounds (TMS compound) were separated on a DB5ms capillary column (30 m x 0.25 mm, 0.25 µm film thickness). The GC oven program: 40 ºC (held for 8 min) to 280 ºC (7 ºC $min^{-1}$) holding time at 280 ºC for 15 min. Helium served as the carrier gas (1.0 mL $min^{-1}$). The GC-MS interface and ion source temperatures were 250 ºC and 200 ºC, respectively. The ion-trap mass spectrometer was operated in the positive impact electronic mode at 70 eV, and the total scan

time was 0.58 s for the m/z 50–650 range; emission current: 250 mA. Automatic mass spectral deconvolution and automated calculation of retention indices (RI) were performed by the AMDIS software. Linear alkanes from polyethylene pyrolysis products were used for the RI calibration [23]. Compounds were identified from the deconvoluted mass spectra by comparison with published MS data. Integrated peaks were normalised, and the yields are expressed as rel. % based on the sum of all detected components (100%). Only identified lignin-derived compounds with yields above 0.5% are listed.

### 2.3 Phenolic content and antioxidant capacity of lignin fractions

The Folin-Ciocalteu method was used to determine the total phenol content in the lignin fractions. To this purpose, 0.5 mL of lignin fraction (0.15 mg/mL) dissolved in dioxane/water (90:10, v/v) was mixed with Folin-Ciocalteu reactive (2.5 mL) aqueous solution (1:10, v/v) and 2 mL of sodium carbonate (7.5% aqueous solution). The mixture was kept for 2 hours before measuring the absorbance at 760 nm in a spectrophotometer. The total phenol content was determined from the calibration curve of gallic acid standard solution (1- 10 mg/L) and expressed as mg of gallic acid equivalent (GAE)/g of lignin (on dry basis).

The antioxidant activity (AA) of lignin was evaluated as the capacity of the lignin to reduce the free-radical DPPH (2,2-diphenyl-1-picrylhydrazyl) [24] using a Shimadzu UV/1800 spectrophotometer. Briefly, 0.1 mL of lignin sample (100, 250 and 500 mg/L) was dissolved in dioxane/water (90:10, v/v) and mixed with 3.9 mL of $6\times10^{-5}$ mol/L DPPH in methanol. The final concentration of lignin was 12.5, 6.25, 2.5 mg/L. A control sample (0.1 mL of dioxane/water (90:10, v/v) and 3.9 mL of $6\times10^{-5}$ mol/L DPPH) was prepared. To isolate variables and collect the maximum AA of each fraction, the absorbances of control and lignin samples were measured at 515 nm after absorbances were stable (between 3 – 5 hours or longer at higher concentration). The AA was calculated according to Equation 1 for the three concentrations of lignin and as expected, increased AA, leads to a more efficient antioxidant.

$$AA, \% = \frac{(A_{control} - A_{sample})}{A_{control}} * 100 \qquad \text{Equation 1}$$

Where: $A_{sample}$ and $A_{control}$ was the absorbance of sample and control respectively.

The results were also given as the minimum amount needed to reduce 50% of the radical DPPH ($IC_{50}$). For each lignin fraction, a plot of lignin concentration (12.5, 6.25, 2.5 mg/L) versus their absorbance were built. According to Equation 2, “y” was normalised by 50% of the DPPH

absorbance ($A_{control}$) so as to obtain the $IC_{50}$. The lower the $IC_{50}$ the higher the antioxidant power.

$$Y = ax + b \qquad \text{Equation 2}$$

where Y is the absorbance of the control sample (DPPH) divided by 2 ($A_{control}/2$), "a" is the slope, "b" is the intercept of each sample equation and "x" is the $IC_{50}$.

2.4 Biologic activity of lignin fractions

The antibacterial activities of the higher yielded fractions 9 and 7, were evaluated against five bacteria: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* ATCC 13076 and *Bacillus cereus* ATCC 11778. These bacteria were placed on the surface Müeller-Hinton Agar (MHA) in a Petri dish and maintained at 37 ºC for 24h. Then, some colonies were picked up, resuspended in sterile saline solution (0.85%) and the suspension was adjusted to 0.5 McF (equal to $1.5 \times 10^8$ cfu $mL^{-1}$). The suspension was homogeneously cast on the MHA plate using sterile cotton swab.

The first test was performed on MHA plate where 4 stainless steel cylinders (7 mm of diameter) for each plate were applied onto the surface of inoculated medium with sterile forceps and they were filled with 200 µl of a solution containing 15 mg $mL^{-1}$ of kraft lignin fraction diluted in dimethyl sulfoxide (DMSO). The pH of lignin in DMSO stock solution was recorded as ~7.4 for both fractions, discarding pH effect over results. Three treatments were also included, pure DMSO without lignin, amoxicillin (0.128 µg $µl^{-1}$) and cephalexin (0.128 µg $µl^{-1}$). The test was performed in triplicate. Plates were incubated for 18 h at 37 ºC and the zone (mm) of the inhibiting bacterium was then measured.

The second test was performed in broth microdilution method in 96-well microtiter plate format to determine the minimal inhibitory concentration (MIC) in the presence of lignin at different concentrations. For this procedure, 50 µl of Mueller Hinton (MH) broth was inoculated in each well. The two last columns were used to growth control: broth with bacterial inoculum and without antibiotic/DMSO and broth without bacterial inoculum and with antibiotic/DMSO. The concentration of the two kraft lignin fractions 9 and 7, ranged from 50 to 0.1953 mg $mL^{-1}$. The antibiotic used as control was amoxicillin in the concentration of 32 to 0.625 mg $mL^{-1}$ (*E. coli* and *P. aeruginosa*) and 5 to 0.0001953 (*B. cereus*, *S. enteritidis*, and *S. aureus*) µg $mL^{-1}$. DMSO was also tested in the concentration ranges of 7.026 M to 0.027 M. Fifty microliters

($1.5 \times 10^6$ cfu $mL^{-1}$) in MH broth was inoculated in each well and incubated at 37 ºC for 18 h. Afterwards, 30 µl of 2,3,5- Triphenyltetrazolium chloride was pipetted into each well, where the pink colour development indicated bacterial growth. Also, 30 µl of each well was inoculated on MHA to verify the bacterium growth in solid medium.

2.5 Cytotoxicity activity of the various lignin fractions

For cytotoxicity assays, approximately 30 mg of each fraction of lignin powder fractionated from pH (3, 5, 7 and 9) was used. Stock solutions of each fraction were prepared in the DMSO as a dilution vehicle (10 mg of each lignin fraction in 1 mL of filtered DMSO). The pH of the stock solutions was recorded as ~7.4 for all fractions. In addition, these stock solutions were further highly diluted in the media for the respective tests; therefore, discarding any pH effect over results. The treatments were carried out in concentrations of 10, 25 and 50 µg / mL of each of the 4 fractions of lignin and the control was done with DMSO as it is the dilution factor. The chosen treatment time was 48 h. The cytotoxicity tests were performed using the MTT and Crystal Violet methods. The absorbance was determined in a microplate reader (Infinite 200, Tecan Group) at 540 nm.

96-well plates were seeded with $8x10^3$ cells / well with 200 µL; using control (DMSO); lignin fractions at 10, 25 and 50 µg / mL; and the standard (blank, only DMSO in MTT method and sodium citrate in crystal violet method). The tumour cells used were Grade IV gliomas U87MG: responsive to treatment with standard chemotherapy (temozolomide - TMZ), T98G: resistant to conventional treatment and Human fibroblasts (non-tumour cells): HDFa (Human Dermal Fibroblast - adult). They were cultivated in DMEM-High Glucose medium with 10% fetal bovine serum (FBS), kept in an incubator at 37º C and 5% $CO_2$.

## 3 Results and Discussion

3.1 Characterization of lignin fractions

Lignin samples were obtained in a fractionation process from a single hardwood kraft black liquor sample; as expected, they present different composition contents, as can be seen at Figure 2. In terms of carbohydrate amounts, they were low and comparable among the fractions. Regarding total lignin content (acid soluble and acid insoluble lignins, ASL+AIL), fractions 5 and 3 presented higher purity, 94.1 and 94.9%, respectively, while fractions 9 and 7 had lower amount of total lignin and increased inorganic compounds. The reduced lignin

quantity can be inferred by the incomplete protonation of the acidic functionalities in lignin at these pH levels, which leads to high amount of ash due to sodium counter ion [25].

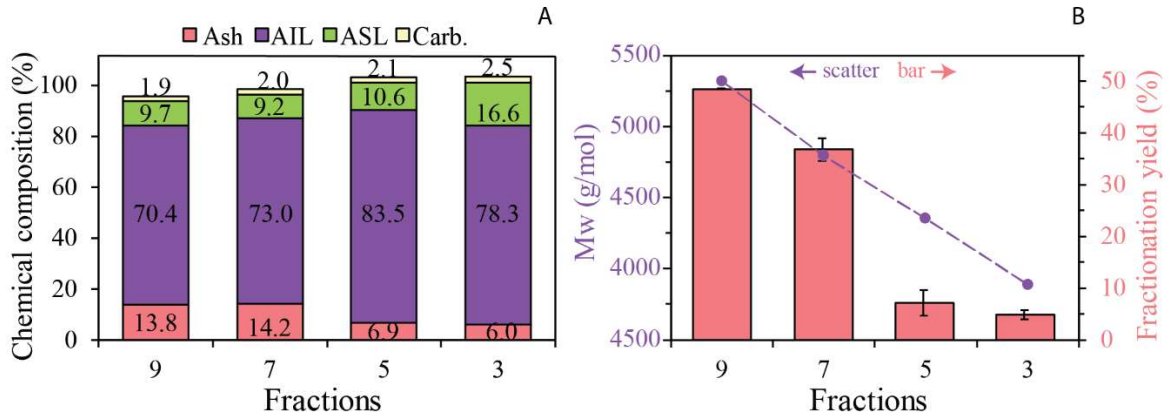


**Figure 2**. Chemical composition (A)* and average molecular weight (Mw)/ fraction yield (B) of lignin fractions precipitated at distinct pH's. Previously discussed in [21,22].
*AIL: acid insoluble lignin; ASL: acid soluble lignin; Carb.; total carbohydrates.

SPyT was used for the characterization of lignin monomers in the fractions [26]. The three types of lignin phenolic units were detected (i.e. hydroxyphenyl, guaiacyl and syringyl, Table 1), but with a predominance of syringyl and guaiacyl units, which were expected since these lignins are obtained from hardwood. In general, kraft process generates lignin with high-condensed structures due to destruction of ether bonds in lignin which leads to the formation of carbon-carbon bonds [27,28]. Such condensed structure can be detected by the presence of non-substituted fragments (guaiacol and syringol), vinyl-, methyl-, and propenyl-substitutes in side chain of phenolic units [26]. The increase abundance of guaiacol and syringol SPyT products may reflect these more condensed structures at higher pH fractions (9 and 7) given a higher Mw (Figure 2). Furthermore, it can be reinforced by the syringyl-to-guaiacyl ratio (S:G) in which the lowest values were presented at higher pH fractions. A higher amount of G-unit allows chemical bond at carbon C5 in the lignin building block indicating a more condensed and stable structure able to be preserved during kraft process [29] (Table 1). Interestingly, the major abundance of guaiacol and hydroxyphenyl units at fraction 9 corroborates with a more condensed structure which may lead to higher Mw. On the contrary, the less condensed structure in that fractions obtained at low pH can be detected by the major abundance of α-carbonyl groups (α-C=O) in the side chains (i.e. aldehydes, ketones and, carboxylic acids groups) leading to a more opened and oxidized structure.

**Table 1.** Relative abundance of SPyT lignin products (as TMS derivative) of sequential acidity decreasing kraft lignin fraction.

| Identity | Class | Fraction | | | |
|---|---|---|---|---|---|
| | | pH 9 | pH 7 | pH 5 | pH 3 |
| C1 - phenol | hydroxyphenyl | 0.6 | - | - | - |
| 4 - hydroxyphenylpropanol | | 1.0 | 0.7 | 0.5 | 0.6 |
| Guaiacol | guaiacyl | 11.0 | 6.4 | 2.5 | 1.9 |
| C1 - guaiacol | | - | - | 1.5 | 1.0 |
| C2-guaiacol | | - | - | 0.7 | - |
| Vanillin | | 1.2 | 2.0 | 2.5 | 0.7 |
| 4 -vinylguaiacol | | 3.2 | 3.7 | 4.7 | 4.1 |
| 4 - hydroxy - 3 - methoxy - phenol | | 2.8 | 3.3 | 0.8 | 0.5 |
| vanillyl alcohol | | - | 0.8 | 1.1 | 0.8 |
| (E)-Isoeugenol | | - | 0.7 | 1.1 | 0.6 |
| Acetovanillone | | 0.6 | 1.2 | 1.3 | 0.7 |
| vinyl alcohol guaiacol | | - | 0.8 | 0.5 | - |
| ferulic acid | | - | - | - | 0.7 |
| dihydroferulic acid | | - | - | 0.6 | 0.0 |
| dihydroferulic acid (isomer) | | - | - | 0.6 | 0.0 |
| C1-Syringol | syringyl | 0.5 | 1.1 | 5.2 | 3.5 |
| Syringol | | 24.0 | 21.0 | 12.4 | 7.0 |
| C2-syringol | | - | 0.7 | 2.1 | 1.1 |
| 4 - vinylsyringol | | 4.0 | 7.6 | 14.5 | 14.3 |
| 4 - allylsyringol | | - | - | 1.3 | 0.9 |
| 4 - hydroxy - 3,5 - dimethoxy - phenol | | 3.9 | 3.6 | 0.5 | 0.5 |
| Syringaldehyde | | 2.8 | 5.8 | 12.0 | 4.1 |
| 4 - propenylsyringol (trans) | | 0.0 | 0.6 | 1.6 | 1.1 |
| Acetosyringone | | 1.6 | 3.0 | 3.7 | 1.7 |
| homovanillic acid | | - | - | - | 0.7 |
| sinapinic acid, methyl ester | | - | - | 0.6 | 0.0 |
| homosyringic acid | | - | - | - | 2.0 |
| sinapinic acid | | - | - | - | 1.3 |
| sinapyl alcohol (trans) | | - | - | 0.7 | 0.0 |
| TOTAL hydroxyphenyl | | 1.6 | 0.7 | 0.5 | 0.6 |
| TOTAL guaiacyl | | 18.9 | 18.9 | 17.9 | 11.1 |
| TOTAL syringyl | | 36.8 | 43.4 | 54.5 | 38.3 |
| S:G | | 1.9 | 2.3 | 3.0 | 3.5 |
| α-CH2 | | 2.1 | 3.3 | 12.3 | 9.7 |
| α-C=O | | 6.2 | 12.0 | 19.5 | 7.2 |
| α-C=C | | 3.2 | 5.0 | 8.7 | 7.1 |

In fact, the less condensed structures at low pH lignin fractions (5 and 3) present, besides the α-carbonyl groups (e.g. vanillin and syringaldehyde), a major relative abundance of α-$CH_2$ (e.g. methyl and ethyl-substituted in the side chain) and α-carbon double bond (e.g vinyl-substituted in the side chain of syringyl/guaicyl units) groups. Furthermore, fraction from pH 3 presented to be more oxidized due to the increase amount of functional carboxyl groups within the side chains (i.e. homovanillic, homosyringic and sinapinic acid, Table 1), which could impart and lead to a lower Mw (Figure 2).

### 3.2 Phenolic content and antioxidant activity (AA) of kraft lignin fractions

The quantification of total phenolic as GAE is widely used for correlation with antioxidant capacity of the investigated material [30,31]. The higher number of phenolic groups for lower fractions (7, 5 and 3) led to significant higher values of gallic acid equivalent (GAE) (Figure 3A). In fact, fractions with high phenolic content presented higher AA (Figure 3B). Moreover, it can be observed an increase over the radical reduction as a function of lignin concentration. At a higher concentration (12.5 mg/L), all fractions presented AA over 50%; whereas, 5 and 3 kraft lignin fractions presented the scavenging activity over 80% (lowest $IC_{50}$ values, Figure 3C). Kraft lignin -without any fractionation process- evaluated with DPPH and same concentration of 12.5mg/L has been reported as presenting 30% of the AA [32]. Same AA value was observed for grass lignin [20]. Another study investigated the AA of different lignin fractions and types at a concentration of 25 mg/L. The AA was similarly measured by DPPH reduction and ranged between 32-64% [33].

When the ash contents were discounted and the value of total lignin (i.e. AIL and ASL, Figure 2A) were computed in the $IC_{50}$ calculation, no differences were perceived between the fractions 3, 5 and 7. In this scenario fraction 7 belongs both to same statistic group as 5 and 3 and fraction 9 (Figure 3D), though fraction 3 and 5 are statistically different from fraction 9.

Factors such as *ortho*-methoxy substitution, and the variety of functional groups presented in the side chain of phenolic units (e.g. guaiacyl and syringyl) from the lignin structure (i.e. methylene and carbonyl groups) may provide antagonist effects on the scavenger activity [31]. The Py-GC-MS method has been used for the investigation of lignins structural properties connected with the scavenger activity [34]; whereas the influence of ortho-methoxyl group (i.e. guaiacyl and syringyl) and methylene (α-$CH_2$) groups in the α-position in the side chain is a positive effect, the influence of the α-carbonyl groups (α-C=O) in the side chain provides a negative effect [31]. Such observations are in accordance with the experimental studies made

with lignin model compounds [31]. It was also observed that double bond (α-C=C) conjugated with aromatic ring shows a depletion in the antioxidant activities as well.

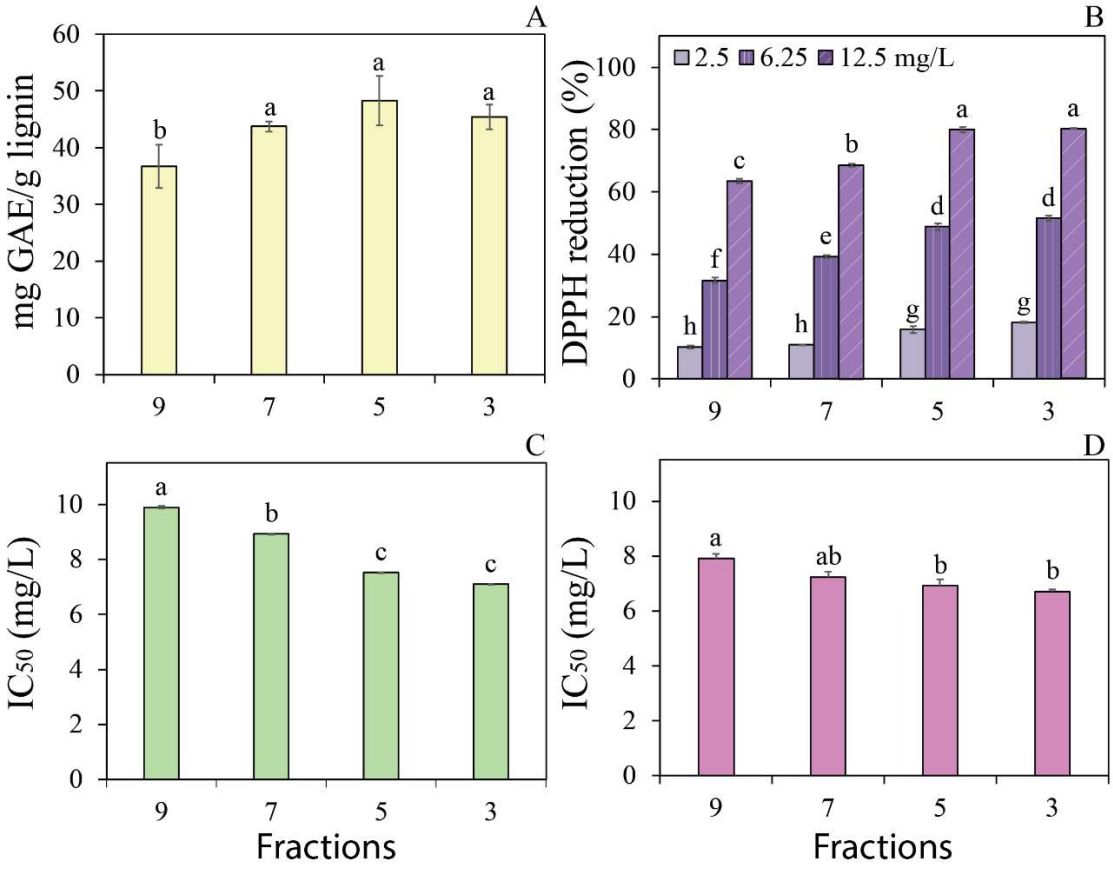


**Figure 3**. Total phenolics as gallic acid equivalent (GAE) (A). Antioxidant capacity of hardwood kraft lignin fractions as DPPH reduction at three different concentrations (B), as $IC_{50}$ values considering the entire chemical composition of each fraction (C) and $IC_{50}$ values corrected by removing the ash content (D). Same letters do not differ statistically by Tukey's test ($p < 0.05$).

Therefore, the high abundance of α-$CH_2$ in the side chain in the fraction 3 and 5, and the major amount of syringyl-type lignin in fractions 3, 5 and 7 are related to a positive effect in scavenger activity. However, the contrary is expected due to the high abundance of α-C=O and α-C=C groups in the fractions 3, 5 and 7 (Table 1). The difference in the AA from the various lignin fractions may be strongly related to their composition (i.e. heterogeneity and purity) [35], and the activation and deactivation of scavenger activity may act simultaneously due to the presence of chemical groups (e.g. α-$CH_2$ x α-C=O). Although, it was found that their ability to scavenge free radicals decreases drastically in the presence of the α-C=O [34], their presence is associated to a more open structure – degraded – which may lead to low steric effects releasing the phenolic groups to react. Interestingly, the α-C=O structure is the main difference between fractions 7 and 9, while the last one is a less oxidized structure with a higher molecular

weight (Table 1, Figure 2), and this has been suggested as a contributing factor to decrease the radical scavenging activity [35]. Furthermore, Figure 3C shows the $IC_{50}$ of lignin fractions decreasing as the total lignin content increased; thus, the presence of inorganics components in the fractions 9 and mainly in 7 (Figure 2) may dilute lignin content, phenolic and other functional groups, and consequently, reduce their AA.

### 3.3 Bioactivity of lignin fractions

Considering the positive antioxidant results and higher yields of lignin from pH 9 and 7, these fractions were selected to be tested against five different bacteria (Figure 4). The lignins exhibited different responses depending on the microbes. Both fractions caused inhibition on *B. cereus* with similar results compared to the standard antibiotic amoxicillin, whereas DMSO does not alter the growing of this specific bacteria. For *P. aeruginosa* and *S. aureus* the inhibition caused by the lignin fractions might be related to DMSO. For *E. coli* and *S. enteritidis* there was no inhibition of bacterial growth by the lignin fractions tested. The growth inhibition observed on *E. coli* by DMSO was not observed in the presence of lignin fractions. The specific amount of added lignin seemed to attenuate the growth inhibition caused by pure DMSO. One hypothesis lies on the possible aggregation of lignin particles providing a micro-environment for growth as an inert solid support, as observed by Wang et al. 2019 [36] for gram-negative bacteria.

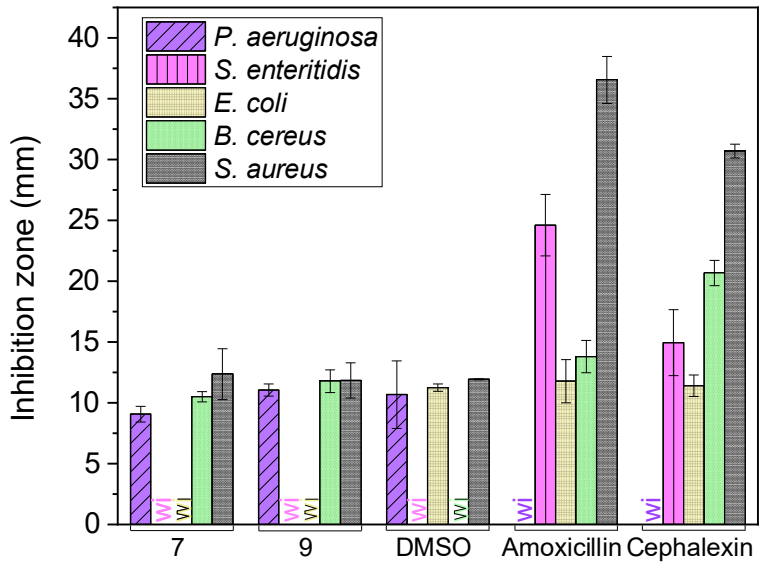


**Figure 4.** Growth inhibition zone (mm) of kraft lignin fractions against different bacteria. wi: without inhibition

The growth inhibition capacities tests, above described, were performed by casting the lignin suspensions on the MHA - inoculating bacteria discs - and measuring the growth inhibition diameter. A different approach, where bacteria were in direct contact with lignin, evaluated the minimal inhibitory concentration (MIC) of lignin (detailed experimental in M&M). The MIC for lignin fractions 9 and 7, amoxicillin and pure DMSO is shown in Table 2. Overall, the two lignin fractions presented a minimal inhibitory concentration against all tested bacteria. The gram-negative bacteria *E. coli* and *S. enteritidis* were the most resistant bacteria resulting in the highest MIC's of lignin fractions. *S. enteritidis* is often reported to be responsible for human infection by food ingestion, mainly fish, shellfish, poultry, eggs and egg-derived products [37,38]. Furthermore, there seems to be a variation on the effectivity depending on the fraction used. Whereas lignin fraction 9 presented the highest MIC on *E. coli*, lignin fraction 7 presented on *S. enteritidis*. It is worth noticing that the pure DMSO tested on all bacteria, presented MIC always above the concentration of DMSO in fact present in the lignin fractions; therefore, the inhibition can be mainly attributed to the lignin.

The less resistant bacteria include one gram-negative *P. aeruginosa* and one gram-positive *B. cereus* in the presence of both lignin fractions (MIC 3.13 mg/mL). *B. cereus* is also associated with food poisoning and is increasingly reported to be a cause of serious and potentially fatal non-gastrointestinal-tract infections [39]. The lower lignin purity, lower syringyl moieties and higher Mw of fraction 9 compared to fraction 7, did not interfered in the antibacterial effects. Both fractions were efficient against both gram-stain. These fractions represent more than 80% of the recovered material, suggesting more realistic applications for a short-term perspective. Moreover, as the lowest and highest MIC were found for gram-negative bacteria in the presence of the lignin fractions, we can infer that the morphology and other bacteria characteristics play a more important role than the stain, when it comes to the resistance to a certain compound.

**Table 2.** Minimal inhibitory concentration (MIC) against different bacteria.

| Bacteria | Fraction pH 9 | Fraction pH 7 | Amoxicillin | DMSO |
|---|---|---|---|---|
| | mg $mL^{-1}$ | | µg $mL^{-1}$ | M |
| *P. aeruginosa* | 3.125 (0.439) | 3.125 (0.439) | 16 | 3.513 |
| *B. cereus* | 3.125 (0.439) | 3.125 (0.439) | ne | 1.756 |
| *S. aureus* | 3.125 (0.439) | 6.250 (0.878) | ne | 1.756 |
| *S. enteritidis* | 6.250 (0.878) | 12.5 (1.756) | ne | 3.513 |
| *E. coli* | 12.500 (1.756) | 6.250 (0.878) | 32 | 3.513 |

ne: no effects; in parenthesis: concentration of DMSO within lignin (in Mols)

Some microbial effects have been reported in the literature but mostly have incremented an inorganic compound as silver nanoparticles to reach this effect [40–42]. However, this type of element can bring damage to the biological environment by the unknown effect of these nanoparticles as it enters the human cell wall types [41]. Herein, antibacterial activity was provided by lignin itself resulted from a simple fractionation process [21]. Therefore, fractions of kraft lignin have effects on the bacterial growth, characterising a strong potential to be applied as an antimicrobial.

### 3.4 *In vitro* cytotoxicity assay in glioma and fibroblast using lignin fractions

*In vitro* cytotoxicity activity of the various lignin fractions was tested by the methods of MTT and Crystal Violet towards three different cell lines: U87MG and T98G (glioma grade IV) and HDFa - Human Dermal Fibroblast of an adult (non-tumour cells) (Figure 5). The use of DMSO (diluting solvent) as a control did not affect the growth of cells and, the values of lignin fractions can be directly related to the effect of lignin. Therefore, the cytotoxicity patterns from these lignin fractions on the various cells are dosage-dependent, in which higher dosages were statistically significant at reducing cell proliferation. Comparing the different tumour cells, the most effective treatment was against the less resistant U87MG which is also reported to be responsive to treatment with standard chemotherapy (TMZ). However, the T98G cell lines, which is resistant to conventional treatment, was also more resistant to all lignin fractions compared to U87MG, though they were also inhibited by lignin presence and statistically significant differences were perceived at higher dosages for fraction 5 and 7. In addition, human fibroblasts (non-tumour cells) were also tested against these lignins and although they were able to induce a slight inhibition, their values were lower compared to the effectiveness against cancer cells. Nonetheless, lignin fractions that were effective at inhibiting cancer cells activity (fractions 5 and 7), presented even at high concentration no harm against non-cancer cells.

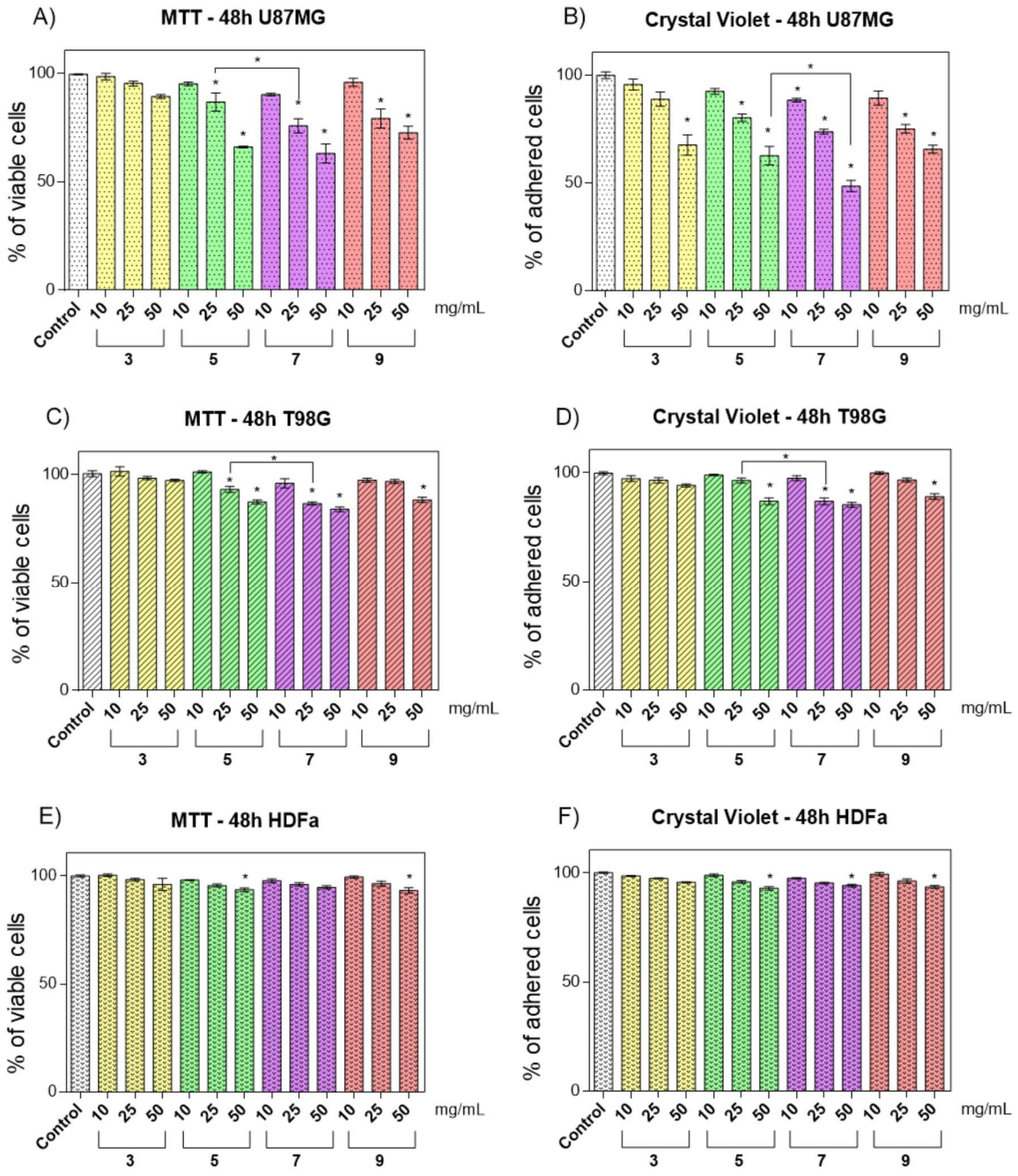


**Figure 5.** Cytotoxicity by MTT and crystal violet methods of glioblastoma cells - U87MG (A, B); T98G (C, D); and non-tumour cells - HDFa (E, F), treated for 48 hours with pH fractioned kraft lignins in concentrations of 10, 25 and 50 mg/mL. The vehicle, DMSO, was used as control and represents 100% of viable or adhered cells in each stain. These data represent the mean ±SD of four independent experiments in triplicate (*p<0,0001).

Previous works have reported little differences between the proliferation of cancerous and non-cancerous cells after kraft lignin treatment [43]. However, the work herein, presents a strong

effect on cancer cells rather than HDFa which could be a potential substitute from conventional treatments. Nonetheless, it is important to state that the exact biological behaviour mechanism of lignin is still uncertain [43]. Some works showed that lignin can inhibit the activation of nuclear factor NF-kb in cancer cells where nearly all cancerous cells have this transcription factor activated which keeps the cell proliferation and prevents cell apoptosis [44]. Another study investigated the interaction of cancerous cells and lignin in maize leaves [45]. It was observed that only hydroxyphenyl and syringyl lignin types were transformed in the presence of the tumour cells. Although these results cannot be directly transferred to human physiology understanding, together, such researches involving lignin and tumour cells work as building blocks towards a more comprehensive knowledge.

## 4. Conclusion

Kraft lignin can be fractionated by acid precipitation to obtain specific molecular weight distribution and chemical compounds which directly influence the antioxidant and biological activities. Overall, the lignin fractions exhibited antioxidant effects attributed to the $\alpha$-$CH_2$ and syringyl-type lignin present majority in the fractions precipitated at lower pH's. The higher yielded fractions 9 and 7 presented both antibacterial effect against all tested bacteria. Moreover, the lignin fractions displayed a positive effect against two glioma grade IV cell lines while only a small cytotoxicity of adult human fibroblasts (non-tumour cells) was detected.

## Acknowledgments

The authors would like to thank CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico (National Counsel of Technological and Scientific Development) for supporting this work. We acknowledge Dr. Bruno D. Mattos for assistance with the illustrations and Otávio Martins Cruz for the support during the antitumoural screening experiments.

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