

Article

Revealing Antioxidant Activity of Cellooligosaccharides and Xylooligosaccharides from Banana Leaves, Pseudostem and Guava Seed Cake

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Abstract

Free radicals are molecules generated during some biochemical processes, and in excess, they can cause various diseases; therefore, their production needs to be controlled in humans. One approach to achieving this is through the consumption of substances with antioxidant capacity, which are capable of neutralizing free radicals. This study evaluated the antioxidant activity of cellooligosaccharides (COS) and xylooligosaccharides (XOS) solutions, extracted from banana leaf and pseudostem, and guava seed cake, unfiltered and filtered using a Sep-pak filter. Additionally, the antioxidant activity of their monomers, including commercial glucose, xylose, and cellobiose, was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay. Antioxidant activity was observed in the unfiltered COS and XOS solutions, with maximum DPPH radical reduction of 48.3% and 84.2%, respectively. In filtered COS and XOS solutions, the reduction did not exceed 0.5%. It can be concluded that the antioxidant activity is due to the presence of compounds dissolved in the oligosaccharide solutions, such as lignin, extractives and carboxylic acids, which were qualified by the Folin–Ciocalteu method, nuclear magnetic resonance, and scanning spectrophotometry.

Keywords: enzymatic hydrolysis; Folin–Ciocalteu; free radicals; scanning spectrophotometry



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1. Introduction

Cellooligosaccharides (COS) and xylooligosaccharides (XOS) are oligomers of glucose and xylan that have the potential to be used as immunostimulants, anticancer agents, prebiotics; for reducing cholesterol and blood glucose levels; and as antioxidants [1]. Antioxidants can be produced by the human body, such as the enzymes coenzyme Q10, glutathione peroxidase, catalase, glutathione S transferase, and superoxide dismutase. Furthermore, they can also be obtained through dietary intake by consuming foods such as fruit, vegetables, and legumes, which contain substances, such as ascorbic acid (vitamin C), vitamin A, tocopherol (vitamin E), polyphenols, selenium, flavonoids, isoprenoids, lycopene, and β -carotene, among others. They can also be industrially produced and added to food, fuel, and cosmetics to prevent oxidation reactions and aid in preservation [2–4]. Antioxidants can inhibit the oxidation of molecules and reduce damage caused by free radicals [5–7].

Free radicals are unstable atoms or molecules that are highly reactive and short-lived due to unpaired electrons in the valence shell and are capable of existing independently.

Due to their instability, they react with other compounds by capturing their electrons and transforming them into free radicals, generating a chain reaction that can cause damage and cell death [2,8]. They are produced by human cells during natural biological processes such as breathing, converting fats into energy, food digestion, and the metabolism of drugs and alcohol [9]. In moderate amounts, free radicals can be beneficial, as they are involved in several biological processes such as the destruction of bacteria by phagocytes and macrophages, cell signaling, the maintenance of the body's homeostasis, and various cellular functions [9,10].

When the human body cannot neutralize free radicals by the natural antioxidant system or when they are produced in excess from sources such as excessive exposure to solar radiation, pollution, certain foods, alcohol, medications, cigarette smoke, and others, oxidative stress occurs. This process leads to cellular and molecular damage, including alterations in deoxyribonucleic acid (DNA), disruptions in normal cell division and energy production. It also affects lipids, proteins, and cell membranes. As a result, oxidative stress can contribute to the development of diseases, such as cancer, neurodegenerative disorders, diabetes mellitus, rheumatoid arthritis, cardiovascular diseases, cataracts, and premature aging [8,9,11–13].

During aging, there is a decrease in the activity of enzymes that act on free radicals; therefore, it is important to consume foods and substances with antioxidant properties [14]. In this context, COS, XOS, and oligosaccharide solutions, in general, have the potential to act as antioxidants, although the exact mechanism is still unknown. Studies with XOS have shown that antioxidant activity is influenced by factors such as the degree of polymerization and the degree of substitution (type and amount of substituent groups in the chain). XOS with uronic acids such as galacturonic or glucuronic acid are more effective at scavenging free radicals. Regarding oligosaccharides in general, the presence of the acetyl group can either enhance or reduce antioxidant activity. Some studies associate this activity with the presence of compounds such as phenols, which are also extracted during the XOS solubilization process [1,15].

To test the antioxidant activity, the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay is one of the most used methods due to its accuracy, simplicity, speed, reliability, cost-effectiveness, and reproducibility [13]. The DPPH method evaluates the concentration of substances with the antioxidant potential required to reduce the initial concentration of DPPH radicals by 50% (IC_{50}). Radical reduction is measured by spectrophotometry before and after adding the studied substances [16,17].

Studies with COS are limited; therefore, the present study aimed to analyze its possible antioxidant activity using the DPPH method. XOS activity was also measured and compared with literature data. XOS and COS solutions were produced using guava seed cake as well as banana pseudostem and leaves, abundant biomasses/waste in Brazil (and tropical countries), pretreated with alkali and enzymatically hydrolyzed (endoxylanase and endoglucanase). The antioxidant activity was measured in both raw hydrolyzed and purified hydrolyzed using a Sep-pak filter to remove colored compounds, phenols, and aromatic substances.

2. Materials and Methods

2.1. Sample Preparation

The guava seed cake was donated by a company called Condire (Dois Corregos-SP, Brazil), after oil extraction. The banana pseudostem and leaves were collected from a local plantation in Rio Claro, SP, Brazil (−22.399754294838946, −47.57176412976766). All biomasses were oven-dried at 60 °C and ground in a knife mill to 20 mesh [18]. This material was then used for pretreatment and enzymatic hydrolysis to produce COS and XOS.

2.2. Chemical Characterization of Biomass and Pretreated Material

Biomass extractives were removed by Soxhlet, washing with water for 8 h, followed by the same procedure with ethanol washing. The material was dried at 60 °C, and approximately 300 mg of extractive-free material was chemically characterized with 1.5 mL of 72% (*w/w*) sulfuric acid for 7 min at 45 °C. After the reaction period, 45 mL of distilled water was added, and the mixture was autoclaved for 30 min at 121 °C, followed by vacuum filtration through a sintered glass filter. The liquid fraction was used to quantify glucose, xylose, arabinose, and acetic acid using high performance liquid chromatography (HPLC) (Item 2.6). The acid-soluble lignin content was determined by ultraviolet-visible (UV-Vis) spectrophotometry at wavelengths of 215 and 280 nm, using 4% sulfuric acid as a reaction blank. The solid fraction was dried at 105 °C (until constant mass) to determine the insoluble lignin content. Total lignin was obtained by adding soluble and insoluble lignin. All tests were performed in triplicate.

2.3. Alkaline Pretreatment

The biomass from the guava seed cake, as well as the banana leaf and pseudostem were pretreated with a 100 mL solution of potassium hydroxide 20% (*w/w*) and autoclaved for 30 min at 121 °C. The solid phase was separated by filtration, washed with distilled water until the pH was close to 7, and dried at 60 °C [19,20]. The material was stored in hermetically sealed bags for further characterization and enzymatic hydrolysis.

2.4. Enzymatic Activity of Endoglucanase and Endoxylanase

Endoglucanase activity was measured by adding 0.1 mL of enzyme cocktail (Celluclast—Novozymes, cocktail rich in endoglucanase) to 0.9 mL of 0.44% carboxymethylcellulose (CMC) substrate in sodium acetate buffer with pH 5.2, with the reaction performed at 50 °C. After 5 min of reaction, 1.5 mL of 3,5-dinitrosalicylic acid (DNS) was added, and the solution was boiled in water for 5 min. Absorbance was measured at 540 nm using a spectrophotometer (Bel, Italy), with distilled water as the blank. For the control, 0.9 mL substrate was subjected to the same reaction conditions, with the enzyme solution added after the DNS. A glucose standard curve was constructed at concentrations of 0.1, 0.25, 0.5, 0.75, 1, 1.5, and 2 g/L to quantify the reducing sugars. The endoglucanase activity was expressed in international units (IU) per mL.

The endoxylanase was produced by *Aspergillus versicolor* growing in wheat bran and purified in a Sephadex G-75 column [21]. Enzyme activity was performed according to [22], with modifications. In a test tube, 1% (*w/v*) Birchwood xylan (Sigma Aldrich, St. Louis, MO, USA), sodium phosphate buffer (pH 6), and a volume of endoxylanase between 10 and 250 µL were added. The tube was heated to 55 °C for 5 min and approximately 250 µL of the reaction mixture was transferred to another tube containing 250 µL of 3,5-dinitrosalicylic acid (DNS) to stop the reaction. To determine the concentration of reducing sugars, a standard curve was made using xylose, and the absorbance was measured with a spectrophotometer at 540 nm. The activity was expressed in units per volume (U·mL⁻¹) of enzyme.

2.5. COS and XOS Production by Enzymatic Hydrolysis

Banana pseudostem and leaves, as well as guava seed cake, were pretreated with 20% (*w/w*) potassium hydroxide. The experimental condition was selected based on a previous preliminary study. Approximately 0.1 g of each material was hydrolyzed with 30 IU·g⁻¹ of endo-β-1,4-xylanase, purified from *Aspergillus versicolor*, in 5 mL of 50 mol·L⁻¹ sodium phosphate buffer (pH 6.0) at a reaction temperature of 55 °C and 170 rpm on a shaker for 24 h [20,21,23]. The tubes were boiled in water for 5 min to stop the enzyme activity. The

liquid fraction was then separated by centrifugation at 5000 rpm for 15 min and filtered through a syringe filter with a pore size of 0.22 μm . Xylose and XOS were quantified in HPLC, according to item 2.7.

About 0.1 g of the solid fraction (residue after endoxylanase hydrolysis) was hydrolyzed with 20 $\text{IU}\cdot\text{g}^{-1}$ of endoglucanase (Celluclast cocktail—Novozymes), with an enzyme activity of 2910 $\text{IU}\cdot\text{mL}^{-1}$, using 5 mL of 50 $\text{mmol}\cdot\text{L}^{-1}$ sodium acetate buffer (pH 5.2) at 50 $^{\circ}\text{C}$ and 170 rpm for 6 h. The tubes were boiled in water, centrifuged, and the liquid fraction was filtered to quantify the glucose and COS by HPLC. The COS and XOS yields were calculated based on the cellulose and hemicellulose content in the raw materials.

The solutions containing XOS and COS were concentrated by lyophilization until approximately 30 $\text{g}\cdot\text{L}^{-1}$. These concentrated XOS and COS yields were further used with appropriate dissolution/dilution for antioxidant activity.

2.6. Determination of Monosaccharides and Acetic Acid

The contents of glucose, xylose, arabinose, and acetic acid from the chemical composition characterization were determined by HPLC using a Bio-Rad Aminex HPX87H (300 $\times$ 7.8 mm) column maintained at 50 $^{\circ}\text{C}$, Waters 2414 refractive index detector at 40 $^{\circ}\text{C}$. The mobile phase consisted of 0.005 $\text{mol}\cdot\text{L}^{-1}$ sulfuric acid at a flow rate of 0.6 $\text{mL}\cdot\text{min}^{-1}$, and sample injection volumes of 20 μL . The samples were filtered using a syringe filter with 0.22 μm pore size. The contents of glucan, xylan, arabinan, and acetyl groups were calculated by multiplying the percentages of glucose, xylose, arabinose, and acetic acid by their hydrolysis factors (0.9, 0.88, 0.88, and 0.72, respectively). The hemicellulose content was reported as the sum of xylan, arabinan, and acetyl groups [24].

2.7. Determination of COS and XOS

COS and XOS concentrations were determined by HPLC using Waters equipment, under the following conditions: Aminex HPX-87C BIO-RAD column (300 $\times$ 7.8 mm); temperature: 80 $^{\circ}\text{C}$; eluent: ultrapure water with 0.6 $\text{mL}\cdot\text{min}^{-1}$ flow; sample volume: 20 μL ; detector: refractive index at 40 $^{\circ}\text{C}$, analysis time: 15 min. Glucose (C1) (Sigma), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5), and cellohexaose (C6) (Megazyme) solutions were used as standards. Samples were filtered using a syringe filter with a 0.22- μm pore size. Xylose (X1) (Sigma), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) (Megazyme) solutions were used as standards [23,24].

2.8. DPPH Free-Radical Scavenging Assay

The analysis was performed according to [25], with modifications. The COS and XOS samples were divided into two groups: unfiltered; and filtered solution with a Sep-pak filter to remove aromatic compounds, phenolics and color substances. Approximately 0.5 mL of glucose, xylose, cellobiose (Sigma-Aldrich), XOS and COS samples at concentrations of: 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 8 and 16 $\text{g}\cdot\text{L}^{-1}$ were mixed with 1 mL of 0.1 $\text{mmol}\cdot\text{L}^{-1}$ DPPH solution in ethanol. The mixture was incubated in the dark for 30 min, and absorbance was measured using a UV-Vis Spectrophotometer at 523 nm. To prepare the negative control, 0.5 mL of ethanol and 1 mL of DPPH solution were used. Ethanol was used as the blank. The scavenging activity was calculated using the following equation:

$$\text{DPPH Scavenging (\%)} = ((A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}) \times 100$$

where

A_{control} : absorbance of the control.

A_{sample} : absorbance of the sample.

2.9. Determination of Phenolic Compounds by the Folin–Ciocalteu Method

To determine the content of phenolic compounds in the samples, the Folin–Ciocalteu method for microplates, with modifications, was used [26]. A volume of 25 μL of each sample (COS and XOS) was added to each well of the microplate, followed by 200 μL of ultrapure water and 25 μL of Folin–Ciocalteu reagent (diluted 1:3). After 5 min, 25 μL of 10% (*w/v*) sodium carbonate was added, and the mixture was kept in the dark for 60 min. After this period, the plate was read using a Tecan SunriseTM (Switzerland) absorbance microplate reader at 760 nm. A calibration curve was prepared with gallic acid at concentrations ranging from 20 to 200 $\text{mg}\cdot\text{L}^{-1}$, and the results were expressed as μg gallic acid equivalents (GAE) per mg of XOS. Reactions were carried out in triplicate, and xylose and glucose solutions were used to evaluate polysaccharide influence.

2.10. Determination of Phenolic Compounds by Scanning Spectrophotometer

Aliquots of the COS and XOS solution were analyzed in a UV–Vis scanning spectrophotometer (Global Analyzer) covering the ultraviolet (200–380 nm) and visible (380–800 nm) ranges, with 5 nm steps. To compare them with the samples, the following solutions were also analyzed: 1% (*w/v*) cellobiose, sodium acetate buffer with Celluclast enzyme, sodium phosphate buffer with endoxylanase from *Aspergillus versicolor* lignin soluble in sulfuric acid (obtained from chemical characterization), extractives removed from banana pseudostem biomass, and 1% (*w/v*) gallic acid.

2.11. NMR Analysis

All NMR spectra were acquired using a Bruker Avance III HD 14.1 T spectrometer (Massachusetts, USA) equipped with a TCI cryoprobe. ^1H NMR spectra were acquired using the zg30 pulse sequence available in the spectrometer's standard library, with a spectral width of 12 kHz and 65,536 data points. The relaxation delay (D1) was set to 1.0 s, and 128 to 158 transients were accumulated to ensure a good signal-to-noise ratio. Data processing was performed with 131,072 points without apodization. $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were acquired using the zgpg30 pulse sequence available in the spectrometer's standard library, with a spectral width of 36.2 kHz, 32,768 data points, and ^1H decoupling during relaxation and acquisition using either the GARP or WALTZ16 pulse train. The relaxation delay (D1) was set to 1.0 s, and 7100 transients were accumulated. Data processing was performed with 131,072 points, applying exponential apodization with a line-broadening (LB) factor of 2.0 Hz.

3. Results and Discussion

3.1. Chemical Composition

The biomass with the highest cellulose concentration was banana pseudostem (48.41%), followed by guava seed cake (37.7%) and banana leaves (24.1%) (Figure 1). In the chemical characterization of banana pseudostem and banana leaves from the studies by de Freitas and Brienzo (2023) [24] and Richard (2024) [27], the cellulose content was reported as 41.9% and 28.7%, respectively, with hemicellulose at 14.8% and 26.2%, and lignin at 14% and 19.8%. In comparison to the present study, cellulose values were similar, lignin values were lower and hemicellulose values varied.

The cellulose percentage increased only when the banana leaves were pretreated with KOH (12% increase), while hemicellulose and lignin percentages increased in all biomasses after KOH pretreatment. During alkaline pretreatment, swelling and reduction in the degree of cellulose polymerization occur, acetyl groups and uronic acids can be removed from hemicellulose, the bonds between lignin and carbohydrates are broken (enhancing

carbohydrate reactivity), and lignin can be partially decomposed into low molecular weight compounds [28].

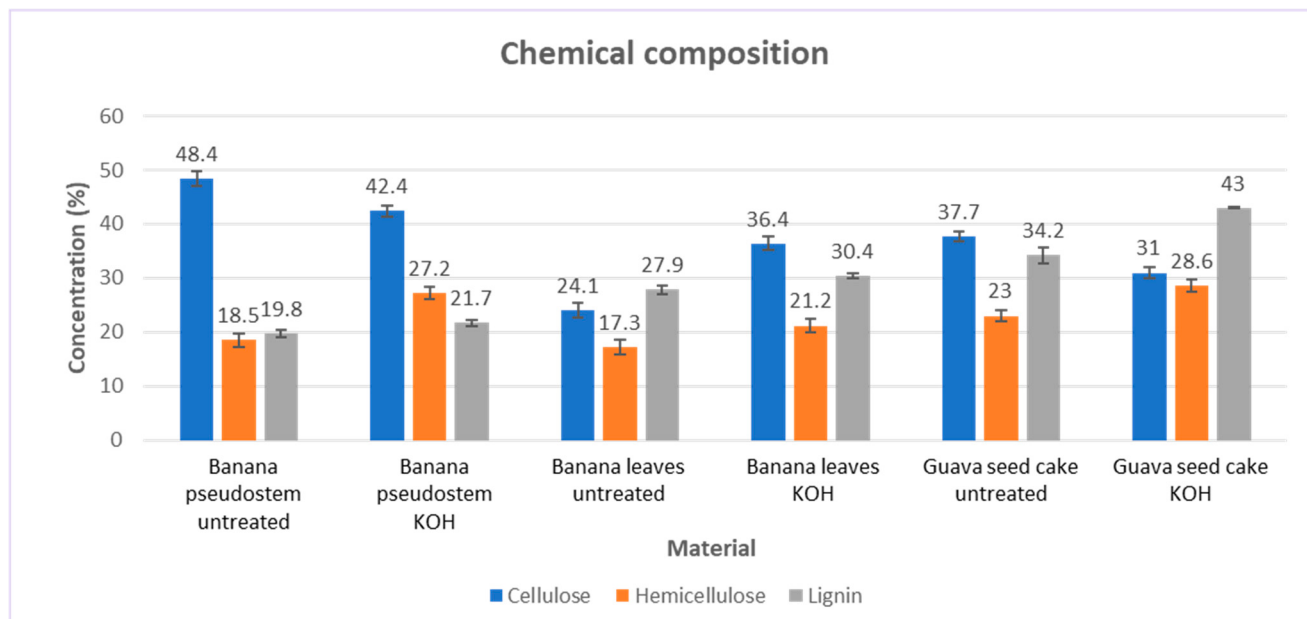


Figure 1. Chemical composition of banana pseudostem and leaves, and guava seed cake, untreated and pretreated.

3.2. XOS and COS Production Yield

The XOS yield was 12.1, 11.5 and 11.6 g·L⁻¹ for banana pseudostem, banana leaves and guava seed cake, respectively, while the COS yield was 4.5, 4.2 and 3.2 g·L⁻¹ for banana pseudostem, banana leaves and guava seed cake, respectively (Figure 2).

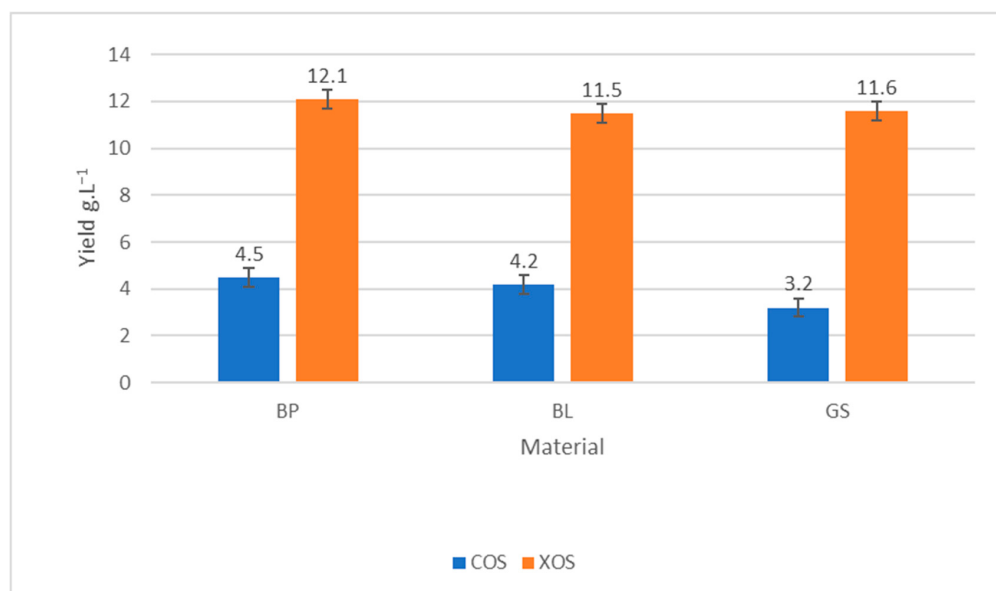


Figure 2. Yield of COS and XOS after enzymatic hydrolysis with xylanase and endoglucanase of the pretreated banana pseudostem and leaves, and guava seed cake bagasse.

During alkaline pretreatment, polysaccharides are not degraded into furfural and hydroxymethylfurfural; however, lignin residues can remain linked to xylan, harming the enzyme activity [29]. To reduce cellulose recalcitrance and increase its accessibility to enzyme action, hydrolysis was first carried out with xylanase before applying endoglucanase.

The removal of hemicellulose by enzymatic action could expose cellulose, increasing its accessibility [30,31]. Moreover, the hypothesis was evaluated considering an increase in the further action of an endoglucanase hydrolysis.

When XOS was produced with rice bran pretreated with NaOH 1% for 1 h at 120 °C and hydrolyzed with xylanase from *Orpinomyces* sp. at 50 °C for 96 h, a concentration of 2.2 g·L⁻¹ was reported, which was approximately five times lower than the yield in the present study [32]. Another study reported a production of XOS using xylan extracted from sugarcane bagasse with 12% (*w/v*) potassium hydroxide, at 121 °C for 45 min and hydrolyzed with endo-1,4-xylanase cocktail 40 U·g⁻¹ of xylan, for 24 h (Novozymes). The concentration obtained was approximately 3.5 g·L⁻¹ [33], which is approximately 3 times lower compared to the present study.

A study reported XOS production using banana pseudostem delignified with 20% hydrogen peroxide (*w/w*), ground in a ball mill for 30 min, and hydrolyzed with endoxylanase from *Aspergillus versicolor* 50 IU·g⁻¹ for 24 h. The result was a concentration of 2.85 g·L⁻¹. In this study, COS was also produced under the same delignification conditions, using the same raw material. The material was hydrolyzed with Celluclast (endoglucanase rich cocktail, Novonesis, Brazil) 50 IU·g⁻¹ for 24 h, resulting in a concentration of 1.26 g·L⁻¹ [24]. In the present study, the XOS and COS yields were approximately 4 and 3.5 times higher, respectively, than in the previous reported study. The conditions most likely used with potassium hydroxide in the pretreatment of banana pseudostem were more effective compared to hydrogen peroxide conditions, making the material more accessible to enzymatic action. In the case of COS production, the hypothesis was confirmed; the addition of endoxylanase before endoglucanase was positive in reducing cellulose recalcitrance.

3.3. DPPH Scavenging Assay

The antioxidant capacity was evaluated by the IC₅₀, which is the concentration of the sample capable of eliminating 50% of the initial DPPH radical. Unfiltered COS and XOS solutions exhibited antioxidant activity, unlike cellobiose and its monomers glucose and xylose (Figures 3 and 4). Moreover, the COS and XOS solutions were filtered through a Sep-pak filter (Figure 5).

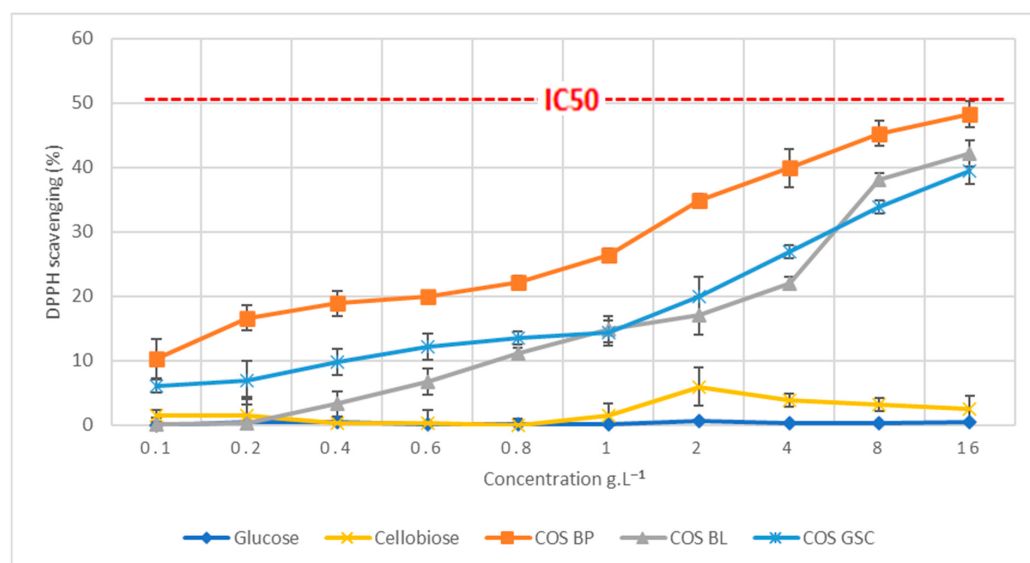


Figure 3. DPPH radical scavenging activity of the glucose, cellobiose and unfiltered celooligosaccharides from banana pseudostem and leaves, and guava seed cake.

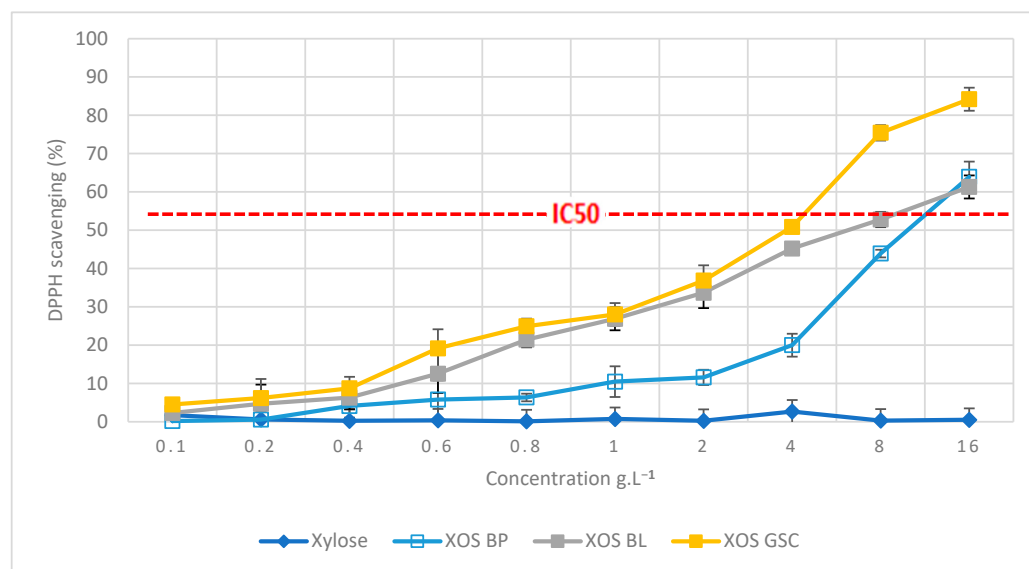


Figure 4. DPPH radical scavenging activity of the xylose and xylooligosaccharides from banana leaves and pseudostem, and guava seed cake.

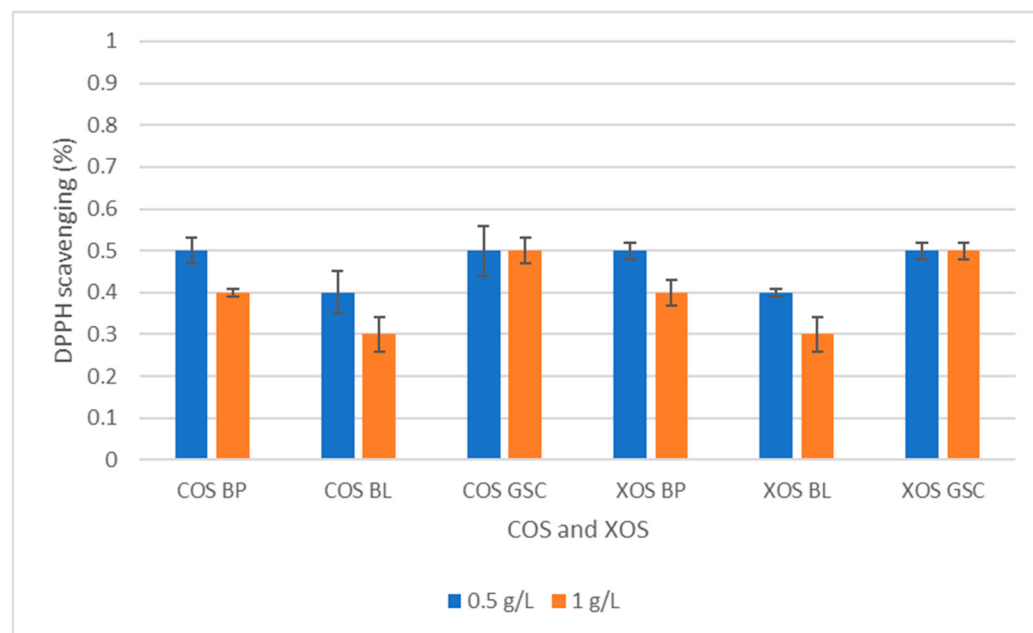


Figure 5. DPPH radical scavenging activity of the cellobioses and xylooligosaccharides filtered through the Sep-Pak filter from banana pseudostem and leaves, and guava seed cake.

In Figure 3 it is possible to observe the values of the reduction in the DPPH radical in percentage. With commercial glucose the maximum value was approximately 0.7%, using a solution of 2 g·L⁻¹. With cellobiose, the maximum value was 5.9% with 2 g·L⁻¹. Regarding unfiltered COS, the IC₅₀ could not be achieved, even using a high solution concentration (16 g·L⁻¹). The highest values of reduction in the DPPH radical were 48.3%, 42.1%, and 39.4% for COS from banana pseudostem, banana leaves, and guava seed cake, respectively.

The maximum reduction of the DPPH radical with xylose solution was approximately 2.7%, with a solution concentration of 4 g·L⁻¹. The IC₅₀ values were obtained in all the XOS solutions, with concentrations of approximately 4 g·L⁻¹ and 8 g·L⁻¹ for guava seed cake and banana pseudostem, respectively (Figure 4).

In the present study, no antioxidant activity was observed with industrial-grade cellobiose with a high degree of purity (98%) (Sigma-Aldrich) (Figure 3). Compared to

the literature, this data indicates that purified oligosaccharides do not have antioxidant activity, except when they are in a solution containing other compounds linked to their structure or in the mixture obtained from hydrolysis [34]. The source of the XOS solution could be responsible for components that act as antioxidants. To confirm this hypothesis, an antioxidant analysis was carried out of the COS and XOS solutions filtered through a Sep-pak filter to remove these compounds. The filtration with this C18 resin filter can remove phenolic compounds, aromatic molecules, lignin derivatives, and degradation products from carbohydrates. As a result, a minimal reduction in the DPPH radical was observed (Figure 5), confirming that the components responsible for antioxidant activities were removed from the COS and XOS solution. The DPPH scavenging with filtered COS and XOS solution did not exceed 0.5% in both cases, compared to 48.3% and 84.2%, with no filtered solution of COS from banana pseudostem and XOS from guava seed cake, respectively.

Unlike COS, there are several studies attributing antioxidant activity to XOS. However, the influence of its chemical structure on antioxidant properties remains unclear and was not properly studied.

In the study by Fuso et al. (2023) [1], the antioxidant activity of XOS and xylose compared to the DPPH free radical was tested. Similarly to the present study, pure commercial xylose did not show antioxidant activity. Commercial XOS with a degree of polymerization of 2 and 6 (Megazyme and Fluka Chemicals), with a purity of 90 and 95%, either showed no reduction or only minimal reduction in the DPPH radical. However, XOS obtained through enzymatic hydrolysis of xylan demonstrated high activity with a degree of polymerization between 6 and 9 xylose units, where IC_{50} was equal to $0.06 \text{ mg} \cdot \text{mL}^{-1}$. Having a degree of polymerization between 2 and 6, XOS showed low activity, not exceeding 38% reduction in the DPPH radical.

Huang et al. (2019) [15] evaluated the DPPH scavenging activity with purified XOS and soluble lignin (SL) obtained from *Moso* bamboo. The increase in activity was directly proportional to concentration and reached 85.7% and 80.7% with a concentration of $2.5 \text{ g} \cdot \text{L}^{-1}$ and $1.2 \text{ g} \cdot \text{L}^{-1}$ for XOS and SL, respectively. Above these values, the activity was stable. IC_{50} values were $0.5 \text{ g} \cdot \text{L}^{-1}$ and $1.1 \text{ g} \cdot \text{L}^{-1}$ for SL and XOS, respectively. These results indicate that SL performs better in scavenging free radicals than XOS, due to the presence of phenolic and aliphatic hydroxyl groups. Using XOS extracted from corn cob with concentrations ranging from 0.2 to $3 \text{ mg} \cdot \text{mL}^{-1}$, the percentage of DPPH radical scavenging ranged from 9.7 to 74.2%. The IC_{50} was $1 \text{ mg} \cdot \text{mL}^{-1}$ [35]. The IC_{50} values obtained by Huang et al. (2019) [15] and Gowdhaman; Ponnusami (2015) [35] were similar and superior to the present study, which had the highest IC_{50} equivalent to $4.1 \text{ g} \cdot \text{L}^{-1}$ with guava seed cake.

The antioxidant activity of oligosaccharides was probably due to the release of soluble or insoluble phenolic compounds, such as ferulic, vanillic, gallic, acid, p -hydroxybenzoic, syringic, p -coumaric, uronic and methyl glucuronic acid, as well as the content of reducing sugars, which can donate electrons to free radicals. Additionally, the antioxidant activity of oligosaccharides can be enhanced by modifying their structure through acetylation and carboxymethylation reactions [34–37].

3.4. Antioxidant Compound Identification

The Folin–Ciocalteu method was used to determine the presence of total phenolic compounds in the COS and XOS solutions, possibly contributing to the reduction in the DPPH radical. In the XOS solution, a higher concentration of total phenolic compounds (TPC) was found concerning the COS solutions (Table 1). XOS from guava seed cake showed the highest reduction in the DPPH radical and the mixture with the highest amount of TPC, followed by the XOS from banana leaves and banana pseudostem, whereby the pseudostem

showed the highest TPC value among these two. Regarding COS, although the IC₅₀ was not reached in any case, it was observed that a greater reduction in the DPPH radical was obtained with COS from pseudostem, leaves and guava, respectively. The TPC value was similar for banana biomass and lower for guava (Table 1).

Table 1. Total phenolic compounds (TPC) in the cellooligosaccharide (COS) and xylooligosaccharide (XOS) solutions and DPPH IC₅₀ values.

Material	TPC (µg GAE/mg COS or XOS)	DPPH IC ₅₀ Values (g·L ⁻¹)
COS BP	3.47 ± 0.21	-
COS BL	3.70 ± 0.39	-
COS GSC	2.61 ± 0.27	-
XOS BP	4.97 ± 0.48	8.0 ± 0.8
XOS BL	3.86 ± 0.14	6.2 ± 0.6
XOS GSC	5.55 ± 0.58	4.1 ± 0.3

(-): did not reach IC₅₀. Experiments were performed in triplicate. BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake, GAE: gallic acid equivalents, DPPH: 2,2-diphenyl-1-picrylhydrazyl.

A UV–Vis spectroscopic analysis was performed with the COS and XOS solutions. In the COS and XOS profiles that absorb at 335 nm and 460 nm, no spectral signals were found (Figure 6, Table 2).

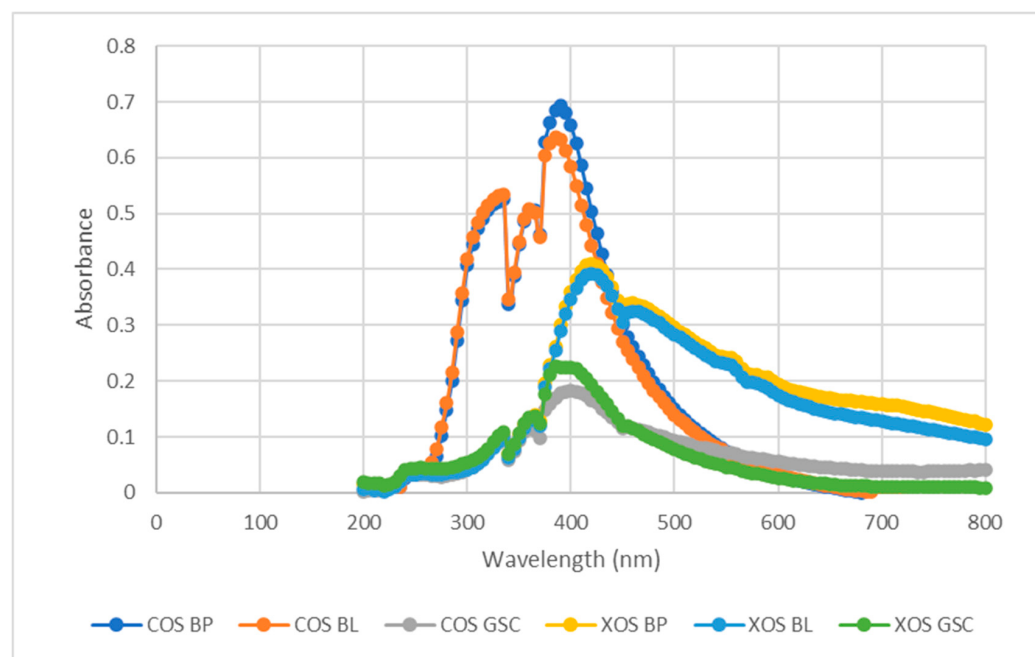


Figure 6. UV–Vis spectroscopic profiles of cellooligosaccharide (COS) and xylooligosaccharide (XOS) from banana pseudostem and leaves, and guava seed cake (200–800 nm). BP: banana pseudostem, BL: banana leaves, GSC: guava seed cake.

Scanning spectrophotometry was carried out on compounds found in the biomass, which may have been transferred to the COS and XOS solutions during the production stages (as no data was found in the literature for comparison). This includes soluble lignin (extracted from banana pseudostem), extractives, and carboxylic acids (represented by gallic acid), which may contribute to antioxidant activity. Additionally, sodium acetate buffer solutions with the Celluclast enzyme and sodium phosphate buffer with *Aspergillus versicolor* endoxylanase, which were used in the enzymatic hydrolysis step, and cellobiose solution, an oligosaccharide formed in greater quantities, were also analyzed (Table 2).

The analysis revealed similar spectral features in soluble lignin, gallic acid and extractive solution samples, possibly due to the presence of carboxylic acids in all solutions.

Table 2. Spectral signals found in samples.

Samples	Spectral Signals (nm)
COS BP	335, 360, 390
COS BL	335, 360, 385
COS GSC	335, 400, 455
XOS BP	335, 420, 460
XOS BL	335, 420
XOS GSC	335, 385
Soluble lignin from banana pseudostem	290, 385, 450, 570, 605
Extractives	315, 360, 385, 570
Gallic acid	290, 385, 450
Sodium acetate buffer + Celluclast	280
Sodium phosphate buffer + <i>Aspergillus versicolor</i> endoxylanase	270
Cellobiose	290, 315

In the COS solutions from pseudostem and banana leaf, and the XOS solutions from guava seed cake, there was probably the presence of extractives and lignin or acid groups (represented for gallic acid in the assay). In the solution of COS from guava seed cake and XOS from banana pseudostem, there have been the presence of lignin or acid groups (gallic acid). This method did not allow for the identification/suggestion of the compounds present in the banana leaf XOS solution.

RMN Analysis

NMR spectroscopy was used to characterize the chemical composition of the obtained COS and XOS to identify compounds potentially responsible for the observed antioxidant activity. The correlation of ^1H and ^{13}C NMR spectra with the literature led to the establishment of relationships between the observed structures and previously identified compounds. Representative NMR spectra of COS and XOS obtained from guava seed cake are presented in Figures 7 and 8, respectively, as examples of the structural features observed. Similar spectral profiles were obtained for COS and XOS produced from banana leaf and pseudostem, reinforcing the presence of common functional groups across the different biomass sources.

The ^1H NMR spectra indicated the presence of functional groups characteristic of polysaccharides, organic acids, and secondary compounds. A prominent signal was observed at 1.9 ppm, which may be associated with acetyl groups bound to oligosaccharides. As reported by Fuso et al. (2023) [1], shifts between 1.87 and 2.28 ppm in ^1H NMR indicate the presence of acetyl groups derived from acetylated xylans. These findings suggest that acetylation may play a significant role in the stability and potential antioxidant activity of the analyzed oligosaccharides.

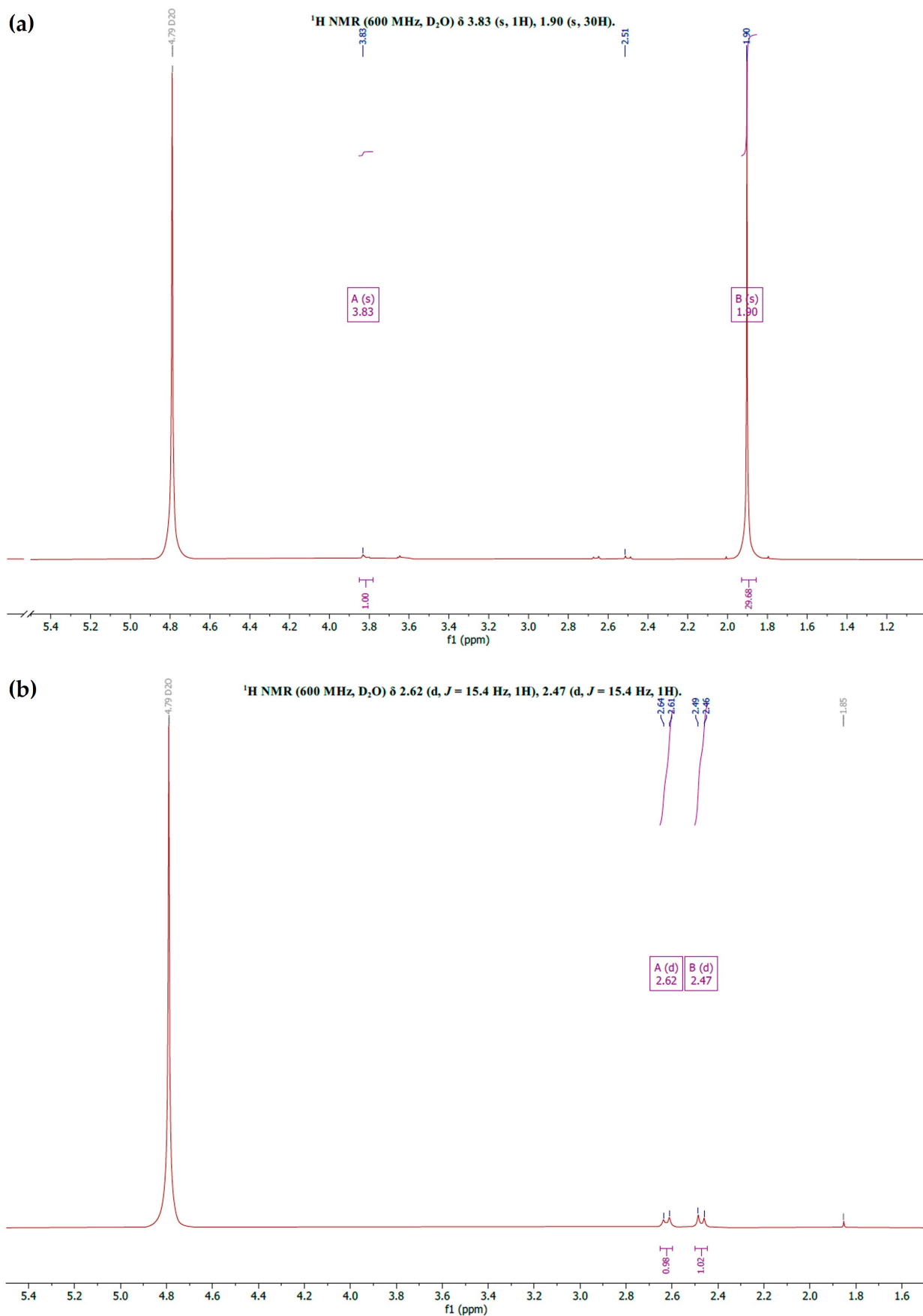


Figure 7. ^1H NMR spectra (600 MHz, D_2O) of (a) cellooligosaccharides (COS) and (b) xylooligosaccharides (XOS) extracted from guava seed cake.

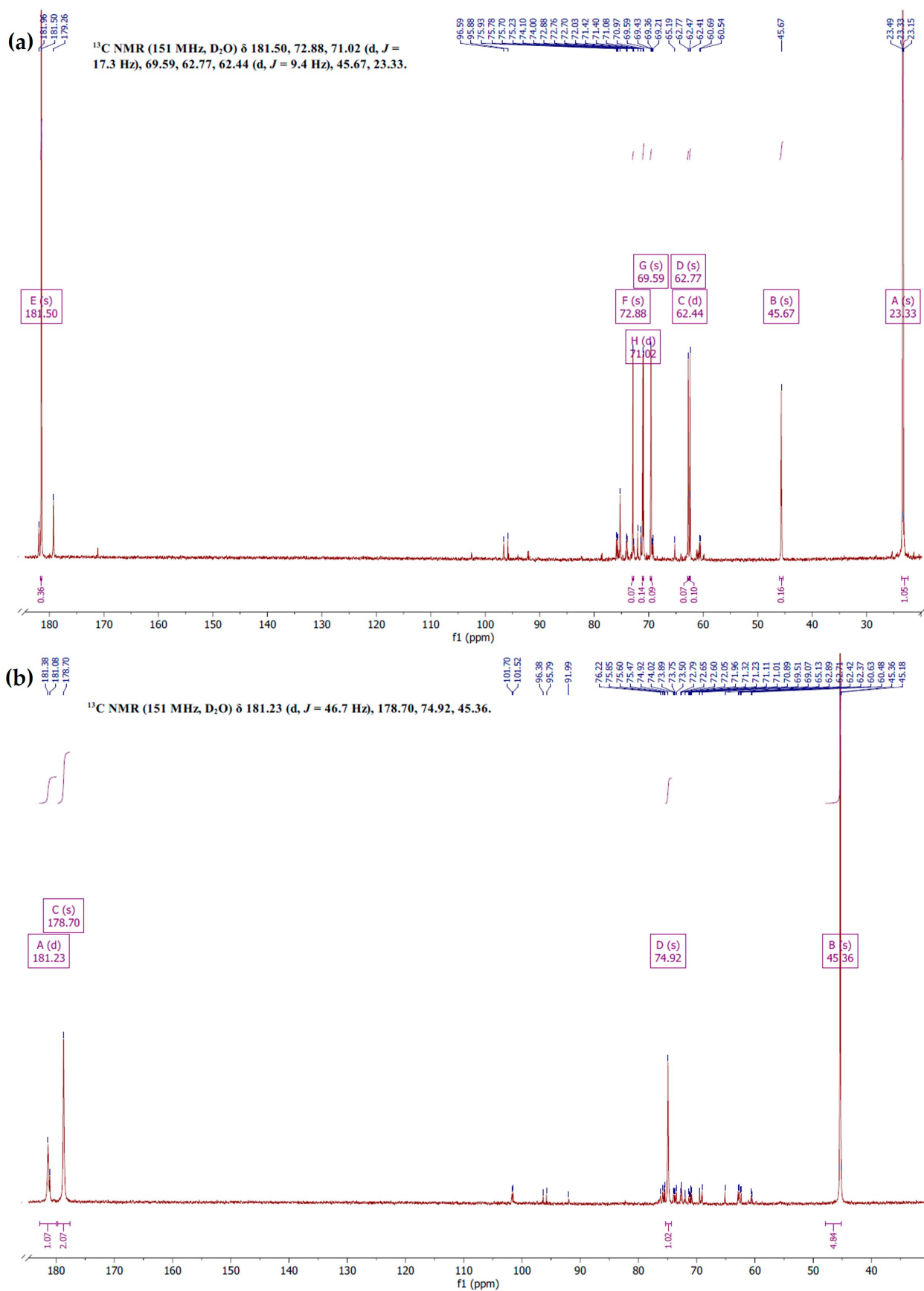


Figure 8. ^{13}C NMR spectra (151 MHz, D_2O) of (a) cellooligosaccharides (COS) and (b) xylooligosaccharides (XOS) extracted from guava seed cake.

Signals observed between 3.95 and 3.16 ppm can be attributed to hydrogen atoms bonded to oxygenated carbons, commonly found in polysaccharides. This is consistent with previous studies where signals in the range of 4.10–3.01 ppm have been assigned to H-2 to H-5 of pentoses, as described by Xu et al. (2023) [38]. Additionally, Bian et al. (2013) [39] identified shifts at 3.19 and 3.47 ppm in XOS spectra, attributed to internal β -D-xylopyranose units, which aligns with the presence of xylan-based oligosaccharides in the samples.

Shifts around 2.69–2.45 ppm suggest the presence of carboxylic acids or related compounds, as previously discussed in studies on organic acid structures in oligosaccharides. Similar assignments have been made in Fuso et al. (2023) [1], where signals between 2.32 and 3.08 ppm were attributed to organic acids, amino acids, and alcohols. The ^{13}C NMR spectra complemented these observations, identifying intense signals between 181 and 179 ppm, attributed to carboxylic acids [40]. The presence of these groups reinforces the hypothesis that carboxylated compounds influence the antioxidant capacity of the samples. Additionally, signals between 77 and 75 ppm were observed, which may be related to lignocellulosic structures, as described by Imman et al. (2021) [41], who identified β -O-4 linkages in oligosaccharides extracted from lignocellulosic biomass. Detecting signals in the 100–103 ppm range suggests the presence of anomeric carbons of xylopyranose residues, while signals at 108–110 ppm can be assigned to terminal or branched arabinofuranosyl units, as described by Sun et al. (2011) [42] in their analysis of arabinoxylans. These assignments further support the structural complexity of the studied oligosaccharides.

The presence of carbonyl and acetyl groups suggests that the observed antioxidant activity may be more related to secondary compounds present in the crude extracts rather than the purified oligosaccharides. The removal of these compounds using the Sep-pak filter resulted in a significant decrease in antioxidant activity, confirming that isolated oligosaccharides exhibit low antioxidant activity. These findings complement the results obtained through Folin–Ciocalteu and UV–Vis analyses, providing a more comprehensive understanding of the mechanisms involved in the antioxidant activity of XOS and COS. A comparison made with the literature reinforces the influence of secondary composition on antioxidant activity, suggesting that the biomass source and extraction processes directly impact the chemical structure of oligosaccharides and their antioxidant potential.

4. Conclusions

COS and XOS solutions produced by enzymatic hydrolysis of the banana pseudostem and leaves, and guava seed cake purified with a Sep-pak filter have a low capacity to reduce the DPPH radical, such as these high-purity commercial glucose, xylose and cellobiose solutions. The Sep-pak filter removes aromatic and phenolic compounds, among others, possibly responsible for the reduction in the DPPH radical. Unfiltered XOS solution has greater antioxidant activity than COS, and a 50% reduction in the DPPH radical was obtained from concentrations ranging between 4 and 8 g·L^{−1}. Using the Folin–Ciocalteu and scanning spectrophotometry methods, the presence of soluble lignin, extractives, and carboxylic acids was suggested in unfiltered COS and XOS samples, which may be responsible for their antioxidant activity. It can be concluded that the production method with endoxylanase and endoglucanase was advantageous for producing COS and XOS simultaneously, and the fact that there is antioxidant activity only with unpurified solutions reduces the need for complex and expensive processes to obtain oligosaccharides with high purity.

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