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Antimicrobial and antioxidant properties of water-soluble lignin extracts obtained from ozonation of *Miscanthus giganteus* and *Vitis vinifera* in a pilot-scale reactor

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ABSTRACT

Lignin is a potential source of polyphenols with different biological activities which might be affected by the extraction or isolation process. This study investigated the effect of ozonation on the bioactive properties of water-soluble lignin extracts from *Miscanthus giganteus* (grass-type biomass) and *Vitis vinifera* (wood-type biomass). Total polyphenolic content, antioxidant activities (ABTS, DPPH and β -carotene bleaching assays), and antimicrobial properties were evaluated. Although ozonation did not improve the antioxidant activity per gram of lignin extract with any of the methods employed, it significantly enhanced extraction of antioxidants. All lignin extracts displayed significant antioxidant potential, DPPH IC₅₀ values ranging from 7.6 to 29.0 μ g/mL and ABTS ranging 64.1–154.6 mg Trolox/g extract. *Miscanthus* lignin extracts achieved 70.6 % β -carotene bleaching inhibition, compared with 96.2 % inhibition by BHT. *Miscanthus* lignin extracts exhibited better yield and antioxidant activity compared with vine shoots, likely due to their lower molecular weight and higher content of phenolic monomers. Antibacterial analysis revealed that *Miscanthus* lignin extracts had superior activity against *Staphylococcus aureus* and *Escherichia coli*, which was enhanced by ozonation and linked to zeta potential and compositional aspects. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Miscanthus* extracts were 3 mg/mL and 6 mg/mL, respectively. *Miscanthus* extracts from 160 min ozonation demonstrated better antifungal activity than vine shoots, particularly against *Penicillium digitatum* (MIC:12 mg/mL) compared with *Botrytis cinerea* (MIC:25 mg/mL). The bioactive properties of water-soluble lignin extracts from ozonated *Miscanthus* open up new possibilities for their potential use in food and food packaging applications.

1. Introduction

Over the last decade, there has been a substantial surge in research into the physiological relevance of bioactive chemicals found in plants (Vinardell et al., 2008). Polyphenols, naturally occurring secondary bioactive compounds sourced from plants, exhibit a diverse range of bioactivities. This heterogeneous group of compounds has received considerable attention from a number of fields, namely food processing, food preservation, and the pharmaceutical sector (Rathod et al., 2023).

Lignin, the second most abundant biopolymer in plants, is a rich source of polyphenols composed of three types of phenylpropane units:

syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) (Eugenio et al., 2021). Lignin's complex structure, which includes aromatic rings containing methoxyl and hydroxyl groups, primarily contributes to its antioxidant potential by terminating oxidation propagation reactions through hydrogen donation. Recent observations regarding the potential toxic and carcinogenic effects of synthetic antioxidants such as propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) when used in high doses, have sparked greater interest in natural antioxidants, including lignin (Aminzadeh et al., 2018). Lignin proves to be a viable substitute for chemically synthesized antioxidants, which are costly to

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produce, energy-intensive, and involve tedious processes (Jiang et al., 2019). Studies have shown that Kraft lignin, organosolv lignin, and lignin fractions from enzymatic treatments exhibit strong antioxidant potential compared with BHT (An et al., 2017; Gordobil et al., 2018; Li et al., 2018). As a result, lignin has been explored as a natural antioxidant in various applications, including food packaging films (Esakkimuthu et al., 2022; Vostrejs et al., 2020; Zadeh et al., 2018), sunscreen and cosmetic products (Antunes et al., 2023, 2023), and biomedical uses (Chao et al., 2023), all showing promising results regarding its antioxidant properties. In comparison to traditional antioxidants, lignin stands out for its environmentally friendly, biodegradable, non-toxic, easily available, and inexpensive nature (Xiao et al., 2021). Moreover, lignin, an essential constituent of the secondary cell wall, serves as a barrier against pathogenic bacteria, boosting plant cell walls' mechanical strength and resistance to pathogen released enzymes that break down cell walls (Li et al., 2023). Considering these properties of native lignin, researchers have explored the antimicrobial activity of lignin, noting its inhibitive effects against some Gram-positive and Gram-negative bacteria, with less effectiveness against fungi (Dong et al., 2011; Gazzurelli et al., 2022; Sinisi et al., 2019; Wang et al., 2019).

Lignin can be extracted from biomass using various methods, including physical, chemical, physicochemical, and biological processes like steam explosion, soda, organosolv, Kraft, and ozonation process. Ozonation is notable for its oxidative nature and high reactivity towards lignin's electron-rich functional groups (García-Cubero et al., 2012). Research shows ozonation effectively degrades lignin, with Li et al. (2021) achieving 40 % degradation of wheat bran lignin, and García-Cubero et al. (2012) observing a 50 % reduction in insoluble lignin from wheat and rye straws. However, the bioactive properties of degraded lignin from biomass after ozonation remain unstudied. The degradation products include phenolic monomers (vanillin, hydroquinone, p-hydroxybenzaldehyde, and syringaldehyde) and organic acids (glyceric, glycolic, oxalic, and malic acids) (García-Cubero et al., 2012; Li et al., 2021), which exhibit antioxidant and antimicrobial activities (Hauser et al., 2016; Zhang et al., 2019). In previous research (Ebrahimi et al., 2024), lignin oligomers, phenolic monomers, and organic acids were identified as lignin degradation products from *Miscanthus giganteus* and vine shoots. *Miscanthus giganteus* produces 18.7–36.8 tons per hectare annually with 15–20 % lignin, while vine shoots yield 15 million tons annually with 24–33 % (David et al., 2020), making both a valuable source for extracting these compounds.

Oligomeric and monomeric phenolic compounds extracted from lignocellulosic biomass through ozone reaction with lignin may possess significant antioxidant and antimicrobial properties (Aminzadeh et al., 2018; An et al., 2019; Neto et al., 2021). While common pH aggressive treatments usually destroy phenolic groups, hampering associated biological activities, ozone might preserve these groups to a higher extent. However, the effects of ozonation on these bioactive properties have not yet been studied. This research studied the impact of ozonation on the bioactive properties of lignin extracts from *Miscanthus giganteus* and vine shoots. Two lignin extraction methods, aqueous and alkaline, were applied to ozonated samples, and efficiency was evaluated by yield increase compared with non-ozonated samples. One extraction method was selected for further analysis of bioactive properties, including total polyphenols, antioxidant (ABTS, DPPH, and β -carotene bleaching methods), antibacterial and antifungal activities. Results focused on the effects of lignin degradation products and ozonation time on these activities. Zeta potential analysis was also conducted to assess its relationship with antimicrobial activity. Among the various applications for lignin extracts, one potential application is as a replacement for sulfite as a food preservative. Therefore, the antimicrobial activity of lignin extracts was compared with that of sulfite.

2. Materials and methods

2.1. Materials

Miscanthus giganteus and *Vitis vinifera* from the pruning of vine trees were used as the initial biomass, sourced from Lamont-Colin Energies (Hangest-sur-Somme, France) and Pago de Carraovejas (Pañafiel, Spain), respectively. *Miscanthus giganteus* was used with particle size smaller than 1 mm, while vine shoots were used with particle size smaller than 1.5 mm for the ozonation process.

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid (97.5 – 102.5 % titration), 6-hydroxy-2.5.7.8-tetramethylchromane-2-carboxylic acid (Trolox), sodium carbonate (≥ 99.0 %), potassium persulfate (≥ 99.0 %), 2,6-Di-tert-butyl-4-methylphenol (BHT), phosphate-buffered saline (PBS), β -carotene, Tween 40, linoleic acid, and chloroform were provided from Sigma-Aldrich (USA). Tryptic soy broth (TSB) and trypticasein soy agar (TSA) were provided from Con-dalab(Spain). Ethanol (96 %) was provided from PanReac AppliChem (Spain).

2.2. Ozonation process

The ozonation process of *Miscanthus giganteus* and vine shoots was conducted in a 0.2 m³ pilot-scale rotary cylindrical reactor, following procedures described in the previous research (Ebrahimi et al., 2024). In this process, 20 kg of biomass was combined with 20 kg of water in the reactor before ozone injection. Ozone was subsequently fed into the reactor at a flow rate of 5 m³/h for 320 min. Samples were taken from the reactor at different ozonation times (0, 40, 160, and 320 min), and subsequently dried for 48 h at 50°C in an oven. Two methods of lignin extraction were applied to the ozonated biomass and the yields of the extracted fractions were compared (Fig. 1).

In the first method, the lignin extracted fractions were obtained by maceration in water, as described in previous research (Ebrahimi et al., 2024). Dried samples were added to water at a ratio of 1:50, shaken in a water bath at 60°C for 30 min, and vacuum filtered. The residue was subsequently dried in an oven, while the filtrate was freeze-dried and stored under dry conditions for further analysis. The lignin extracted fractions' yield (OZ-WE) was determined using Eq. (1).

In the second method, the lignin extracted fractions were obtained through alkaline treatment of the ozonated biomass. In this process, 4 g of biomass was mixed with 40 mL of 1.5 % NaOH and shaken in a water bath at 60°C for 60 min. Following the treatment, the samples underwent vacuum filtration to separate the residue from the filtrate. The lignin extracted fractions' yield (OZ-ES) was determined using Eq. (1).

$$\text{Yield\%} = \frac{(\text{Initial dry ozonated biomass} - \text{residue})}{\text{Initial dry ozonated biomass}} \times 100 \quad (1)$$

2.3. Physicochemical characterization

In a previous research, the effect of different ozonation conditions on the pH, molecular weight and composition of the lignin extracts was studied (Ebrahimi et al., 2024) but a detailed evaluation of the potential antioxidant and antimicrobial properties, which can infer into potential applications, has not been done for water soluble lignin extracts, to the best of our knowledge. Therefore, soluble extracts obtained after different ozonation treatments were evaluated for their antioxidant, antibacterial and antifungal properties, following several approaches, as to deliver a deeper insight into structure activity and potential use. Antioxidant activity in lignin or lignin derivatives can usually be related to their total polyphenol content.

The total polyphenols content (TPC) of the lignin extracts was analyzed using a modified Folin-Ciocalteu method based on Singleton et al. (1999). For this purpose, 5 mL of 80 % ethanol (v/v) was mixed

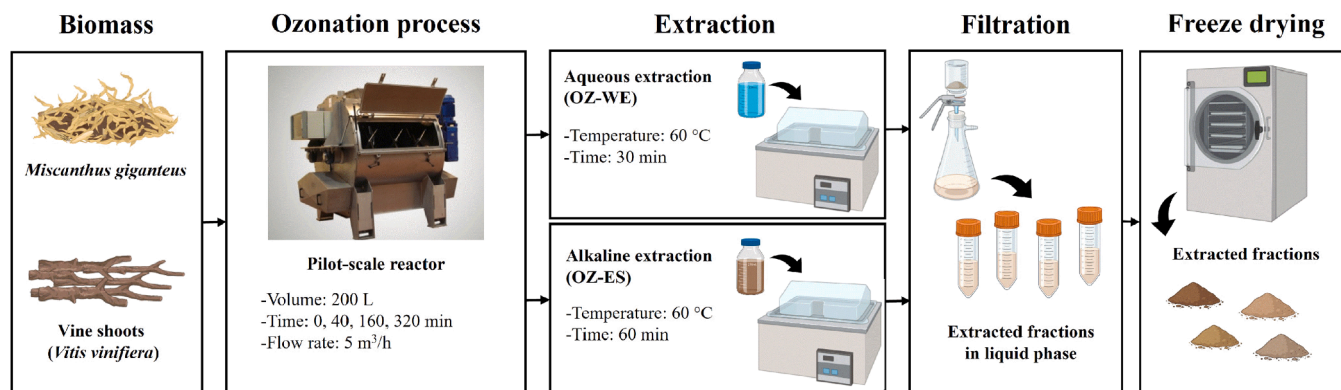


Fig. 1. Schematic illustration of lignin extraction process from *Miscanthus giganteus* and vine shoots, using ozonation process combined with aqueous or alkaline extraction.

with 10 mg of each lignin extract, and the sample was stirred overnight. Subsequently, 7.5 mL of ultra-pure water was added to 1 mL of the sample in triplicates. Following this, 0.5 mL of Folin-Ciocalteu reagent was added to the samples, and the tubes were left in the darkness at ambient temperature for 10 min. Next, 1.5 mL of 20 % (w/w) sodium carbonate was added to the samples, which were then incubated in a water bath at 40 °C for 20 min. The samples' absorbance were determined at 755 nm. Gallic acid was utilized as a standard to calculate total polyphenols, with results presented as milligrams of gallic acid equivalents (GAE) per gram of lignin extract and per gram of biomass. The TPC results based on biomass were calculated by incorporating the extract yield using Eq. (2).

$$\text{TPC}(\text{mg GAE/g biomass}) = \frac{\text{TPC}(\text{mg GAE/g extract}) \times \text{Yield}}{100} \quad (2)$$

2.4. Antioxidant activity

2.4.1. ABTS protocol

The ability of the lignin extracted fractions to reduce the ABTS radical was assessed following the ABTS antioxidant technique as stated by [Fontes-Candia et al. \(2019\)](#). ABTS (0.192 g) and potassium persulfate (0.033 g) were mixed in 50 mL of pH 7.4 PBS and stirred overnight. Before analysis, PBS was added to the ABTS monocations (ABTS•+) solution to achieve a 0.70 ± 0.02 initial absorbance at 734 nm. Then, 230 μL of ABTS monocations solution was combined with 20 μL of each sample in a 96-well plate. After that, the plate was placed in the darkness at ambient temperature for 6 min, followed by absorbance measurement at 734 nm using the CLARIOstar spectroscopy equipment (BMG LAB-TECH). Trolox was chosen as a calibration standard, and the antioxidant capacity of the samples was expressed as mg of Trolox equivalents per gram of sample. Every determination was made in triplicate.

2.4.2. DPPH protocol

The antioxidant property of the lignin extracted fractions was analyzed using the DPPH technique with some modifications ([Scherer and Godoy, 2009](#)). Lignin extracts were mixed with 80 % ethanol to have concentrations of 0, 0.1, 0.2, 0.4, and 0.6 mg/mL. 1 mL of 0.18 mmol/L DPPH methanol-prepared solution (with absorbance of 0.6–0.7 at 517 nm) was mixed with the samples. After one hour of incubation in darkness at ambient temperature, the samples' absorbance was determined at 517 nm. The results were expressed as IC₅₀, which is the minimum concentration of the antioxidant sample required to reduce 50 % of the DPPH radical. Trolox was utilized as a standard to compare the antioxidant activity of the lignin extracted fractions.

2.4.3. β -carotene protocol

The β -carotene bleaching test followed [Miraliakbari and Shahidi's \(2008\)](#) approach, with some adjustments. A mixture of 50 mg linoleic acid and 400 mg Tween 40 was combined with 2 mL of β -carotene in chloroform (0.4 mg/mL) to form a stock solution of β -carotene and linoleic acid. Then, the chloroform was evaporated from the mixture, and 50 mL of aerated water was introduced to the residue. A BHT solution (2 mg/mL) was used as the positive control, and the samples were analyzed at the same concentration. Then, 10 μL of sample, the positive control (BHT solution), and the negative control (distilled water) were dispensed in a 96-well microplate in triplicate. Then, 250 μL of the β -carotene/linoleic acid solution was added to each sample in the plate. The plate's absorbance was determined at 470 nm at time zero, then the plate was placed at 45 °C for 120 min, and the absorbance was determined once more following incubation. The antioxidant activity was measured using the following Eqs. (3) and (4), where Abs₀ is the absorbance at time 0, Abs₁₂₀ is the absorbance after 120 min, DR_{control} is the DR value of the water, DR_{sample} is the DR value of the sample, and AA % is the antioxidant activity:

$$\text{DR} = \text{Ln} \frac{\text{Abs}_0}{\text{Abs}_{120}} \times \frac{1}{120} \quad (3)$$

$$\text{AA\%} = \left(\frac{\text{DR}_{\text{control}} - \text{DR}_{\text{sample}}}{\text{DR}_{\text{control}}} \right) \times 100 \quad (4)$$

2.5. Antibacterial activity

The antibacterial activity of lignin extracts was analyzed against *Staphylococcus aureus* (CECT 59; NCIMB 6571) (Gram-positive), and *Escherichia coli* (CECT 45; NCIB 86) (Gram-negative). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lignin extracts were determined via a broth microdilution technique in sterile 96-well plates ([Wiegand et al., 2008](#)). Samples were sterilized with 80 % ethanol. Different concentrations (25, 12, 6, 3, 1.5, 0.6, 0.4 mg/mL) of the samples were prepared in 10 % TSB for *S. aureus* and 1 % TSB for *E. coli*. Each solution (90 μL) was added to 96-well plates in triplicate. Bacteria with an optical density of 0.2, corresponding to the exponential phase, were inoculated into the wells (10 μL), resulting in an initial concentration of 10^6 CFU/mL. Plates were incubated in an incubator cabinet (Equitec Eris 855HR4500) at 37 °C with a relative humidity (RH) of 75 % for 20 h. Optical density was measured every 15 min at 600 nm using a Spectrostar Omega (BMG Labtech) reader and a Stacker (BMG Labtech) to monitor the growth pattern of each bacterial strain. After 20 h, viable counts were performed on agar plates to determine the MBC. One experiment was performed using pure Klason lignin. A control was conducted using only the medium, while a positive control used sulfite, a common food preservative. A negative

control was added with only bacterial inoculum (no antimicrobial).

2.6. Antifungal analysis

The antifungal property of the lignin fractions was evaluated against *Penicillium digitatum* (Pers.:Fr.) Sacc isolate Pd1 (CECT 20795) (Marcet-Houben et al., 2012) and *Botrytis cinerea* strain B05.10. Conidial suspensions were prepared by applying 5 mL of sterile distilled water to the surface of 7–12-day-old potato dextrose agar (PDA) cultures and scraping with a sterile spatula. Conidia were counted in a hemocytometer and diluted to the desired concentration. The MIC of the lignin extracts was measured using the broth microdilution technique in a 96-well plate (Rodríguez-Tudela et al., 2008), with some modifications. Lignin extracts were sterilized using ethanol 80 %, and different concentrations (0.4–25 mg/mL) were prepared using 20 % potato dextrose broth (PDB). 180 μ L of each sample were incubated with 20 μ L of *Penicillium digitatum* with a concentration of 10^6 conidia/mL or *Botrytis cinerea* with a concentration of 10^5 conidia/mL in 96-well microplates at 24°C for 5 days, and optical density was measured at 600 nm every 120 min to monitor the growth curve of each fungus. Blank experiments were conducted without fungal inoculation to monitor sample contamination. A positive control was conducted using sulfite, a common food preservative, while a negative control included only the fungal inoculum. MIC of each sample was determined based on the fungal growth curve and microscopic images of the cells with lignin extracts, captured using an inverted microscope Eclipse Ts2 (Nikon Corporation, Japan), after 7 days of incubation.

2.7. Zeta potential analysis

The surface charge of lignin extracted fractions was determined by measuring their zeta-potential using a Malvern Nano Zetasizer (UK). Samples with concentration of 5 mg/mL in ethanol were used for average zeta potential analysis.

2.8. Statistical analysis

The statistical analysis of the results was done using a one-way analysis of variance (ANOVA) in Statgraphics Centurion XVII-X64. The significance of the mean values was determined using Tukey-test, at a confidence level of 95 % ($p < 0.05$).

3. Results and discussion

3.1. Ozonation products

Alkaline extraction (OZ-ES) of ozonated *Miscanthus* and vine shoots

consistently produced higher yields than aqueous extraction (OZ-WE) at all ozonation times (Fig. 2). However, the difference between alkaline and aqueous extraction yield decreased with prolonged ozonation. Zhang (2021) reported that alkaline extraction enhances the solubility of oxidized lignin after ozonation by breaking ester bonds between lignin and xylan, while preserving cellulose. Therefore, longer ozonation could further increase ester bond breakage.

The efficiency of ozonation in improving the extraction yield was more significant with the aqueous extraction of ozonated biomass. For *Miscanthus*, the extract yield after OZ-WE treatment increased from 2.92 % to 24.55 % (an 8.4-fold increase), whereas OZ-ES treatment only increased it from 18.12 % to 30.59 % (a 1.7-fold increase). Similarly, for ozonated vine shoots, OZ-WE treatment improved the yield from 4.08 % to 23.88 % (a 5.9-fold increase), whereas OZ-ES treatment increased it from 13.57 % to 27.77 % (a 2.0-fold increase) (Fig. 2). The difference in yield enhancement between *Miscanthus* and vine shoots may come from the initial structure of lignin and the size of particles used for the ozonation process. Syringyl groups in lignin structure are more reactive to ozone than guaiacyl groups (Quesada et al., 2002). *Miscanthus giganteus* has higher syringyl units (44 %) compared with vine shoots (26 %) (Ebrahimi et al., 2024), which results in greater reactivity to ozone, giving higher yield.

Aqueous extraction was chosen as the preferred method for obtaining degraded lignin from ozonated biomass due to its superior improvement in extract yield and as it is an environmentally friendly process. The physicochemical characteristics of these lignin extracted fractions, including structural modification using FTIR analysis, molecular weight analysis using HPSEC-MALLS, phenolic monomers and organic acids analysis using GC-MS, and the thermal stability analysis using TGA were comprehensively detailed in a previous article dealing with the optimization of ozonation process parameters (Ebrahimi et al., 2024). A summary of these physicochemical properties of the lignin extracts is presented in TableS1.

3.2. Antioxidant activity

Antioxidant assays are categorized into two mechanisms: hydrogen atom transfer (HAT), in which radicals are quenched by the antioxidant donating a hydrogen atom, and single electron transfer (SET), where the antioxidant reduce a compound by transferring a single electron (Alzagameem et al., 2018). The usefulness of indirect methods to evaluate antioxidant activity in vitro (ABTS, CUPRAC, DPPH, FRAP, ORAC) is limited, due to the difficulties in translating these results with direct antioxidant performance with real sample matrices or with a real biological activity (Harnly, 2017). In the present work, the antioxidant activity of the lignin extracted fractions from *Miscanthus* and vine shoots was assessed using four different methods, each operating through

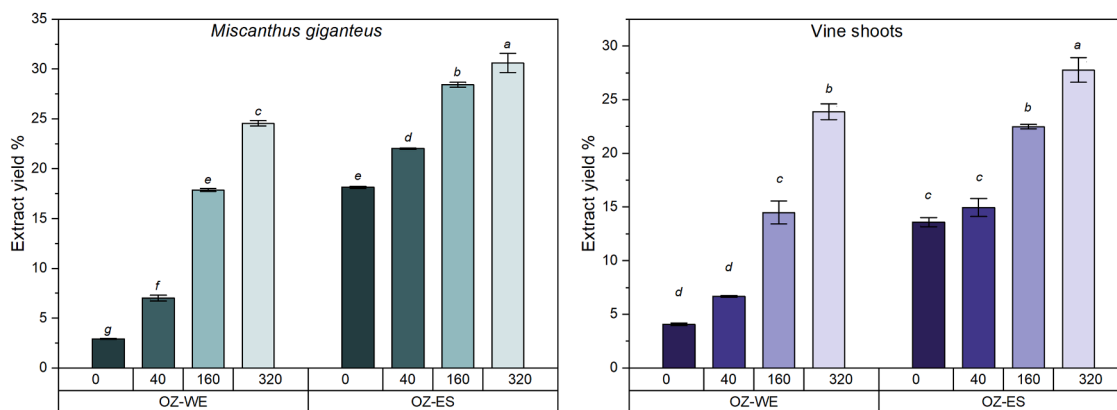


Fig. 2. Extract yield comparison between aqueous extraction (OZ-WE) (Ebrahimi et al., 2024) and alkaline extraction (OZ-ES) for ozonated *Miscanthus giganteus* and vine shoots at different ozonation times (0, 40, 160, 320 min). Different letters indicate significant differences between the mean values at $p < 0.05$ using Tukey-test.

different mechanisms. The Folin-Ciocalteu method, commonly employed for total polyphenols analysis, operates based on the single electron transfer (SET) mechanism. Both ABTS and DPPH antioxidant analysis involve both single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms. Finally, the β -carotene bleaching assay is a direct method for quantifying antioxidant activity in fats and oils, which specifically follows the HAT mechanism (Prior et al., 2005). A comparison of these results with the biological activity (e.g. antibacterial, antifungal) activity of the lignin extracts aimed at enriching this discussion.

3.2.1. Total polyphenols

Total polyphenol content (TPC) results were presented in two ways. First, TPC was shown per gram of lignin extract to examine the effect of ozone on the polyphenol contribution in the lignin extract. Second, TPC was expressed per gram of ozonated biomass, aiming to assess the overall impact of ozonation on polyphenol extraction (Table 1).

Table 1 shows that the TPC of lignin extracts from vine shoots decreased from 51.05 to 28.84 mg GAE/g extract as the ozonation time increased. In contrast, for *Miscanthus* extracts, the TPC initially increased from 31.38 to 42.17 mg GAE/g lignin extract during the first 40 min of ozonation, followed by a subsequent decline. Despite the decrease in TPC/g lignin extract with ozonation time, ozonation generally enhanced polyphenol extraction. For *Miscanthus*, the total polyphenols increased 5-fold from 0.92 to 4.46 mg GAE/g dry biomass, while for vine shoots, they rose from 2.11 to 6.99 mg GAE/g dry biomass after 320 min of ozonation (Table 1). The maximum TPC for *Miscanthus* was reached at 160 min and remained stable thereafter, whereas for vine shoots, significant polyphenols release started only after the first 40 min of ozonation. The differences in TPC between lignin extracts from *Miscanthus* and vine shoots can be attributed to their different cell wall structure. Additionally, the positioning of hydroxyl groups in phenolic compounds influences their ability to scavenge free radicals (Pérez et al., 2023), which could contribute to the difference in TPC between vine shoots and *Miscanthus* lignin extracts.

While no studies have specifically reported the effect of ozone on the TPC of lignin, many researchers have documented the TPC of lignin obtained through other processes. Sheng et al. (2022) reported that total polyphenols of ethanol organosolv lignin from poplar, pine chips, walnut, and rice husks ranged from 8.95 to 10.20 mg GAE/g lignin. Another study by Qazi et al. (2017) showed that pyrolytic lignins exhibited a broader range of total polyphenolics content varying from 0.42 to 50 mg GAE/g of pyrolytic lignin.

Previous studies have also reported results on the effect of ozone on polyphenol extraction from food sources, ranging from improvement to negative or no effect. Jackowska et al. (2019) observed a 24 % and 32 % increase in TPC after ozonation of rapeseed, while Brodowska et al. (2015) reported a 41.7 % loss in TPC after ozonation of cardamom seeds. The ozonation of cow pea resulted in both an 18–40 % increase and no effect on TPC in two different studies (Gad et al., 2021; Malaiyandi and Natarajan, 2014). However, no studies have specifically

Table 1

Total polyphenols of ozonated *Miscanthus giganteus* and vine shoots and their water-soluble lignin extracts at different ozonation time.

Ozonation time (min)	TPC (mg GAE/g lignin extract)		TPC (mg GAE/g biomass)	
	<i>Miscanthus giganteus</i>	Vine shoots	<i>Miscanthus giganteus</i>	Vine shoots
0	31.38 ^e ± 1.35	51.05 ^a ± 0.97	0.92 ^f ± 0.04	2.11 ^e ± 0.04
40	42.17 ^b ± 0.98	27.77 ^c ± 0.81	2.90 ^d ± 0.03	1.86 ^e ± 0.03
160	24.89 ^{cd} ± 4.20	33.51 ^c ± 3.51	4.43 ^e ± 0.12	5.02 ^b ± 0.14
320	18.26 ^d ± 1.58	28.84 ^c ± 1.00	4.46 ^e ± 0.05	6.99 ^a ± 0.04

examined the effect of ozone on the TPC of lignocellulosic biomass. This study demonstrates the positive effect of ozone on polyphenols extraction from two types of biomasses. The increase in TPC with prolonged ozonation time is attributed to ozone reacting with the lignin structure in the biomass, extracting it as polyphenolic compounds. These compounds comprise a mixture of phenolic monomers (phenolic aldehydes and phenolic acids) and lignin oligomers, as described in the previous work (Ebrahimi et al., 2024).

3.2.2. ABTS antioxidant activity

Fig. 3 presents the ABTS antioxidant activities of lignin extracted fractions from both biomasses, expressed as mg of Trolox per g of lignin extracted fraction. *Miscanthus* lignin extracts showed higher ABTS scavenging capacity compared with the vine shoots lignin extracts. With ozonation, the ABTS⁺ free radical scavenging activity of *Miscanthus* lignin extracts decreased from 154.6 to 79.3 (mg Trolox/g extract), whereas for vine shoots lignin extracts, it dropped from 113.6 to 64.1 (mg Trolox/g extract). ABTS antioxidant activity of technical lignins has been widely reported. Lignin extracts obtained by steam explosion or ultrasound-assisted extraction of sugarcane bagasse showed ABTS antioxidant activities in the range of 35 – 83 mg Trolox/g extract (Juttuporn et al., 2018), which is lower than that of the *Miscanthus* and vine shoots extracts in this study. On the other hand, the purified lignin from black liquor of soda/anthraquinone (soda/AQ) pulping of orange and olive tree pruning residues exhibited ABTS antioxidant activities of 149.7 and 598.2 mg Trolox/g lignin respectively, which is higher than the values for *Miscanthus* and vine shoots extracts, likely due to the lignin purification degree. In another study, acetic acid lignin and milled wood lignin from bamboo shoot shell showed 1441.41 and 819.04 μ M Trolox/g lignin (Gong et al., 2016).

Miscanthus and vine shoots lignin extracts have different structural composition influencing their antioxidant properties. Each extract is composed of the mixture of lignin oligomers, phenolic monomers, and organic acids, which can positively or negatively influence the antioxidant activity. According to Zheng et al. (2021), lignin with lower weight-average molecular weight (Mw), number-average molecular weight (Mn) and polydispersity value (Mw/Mn) exhibits higher scavenging ability for reactive oxygen species (ROS). Lignin oligomers in *Miscanthus* extracts have lower molecular weight and polydispersity compared with those in vine shoots (Table S1), resulting in higher ABTS antioxidant activity. Furthermore, the higher syringyl to guaiacyl (S/G)

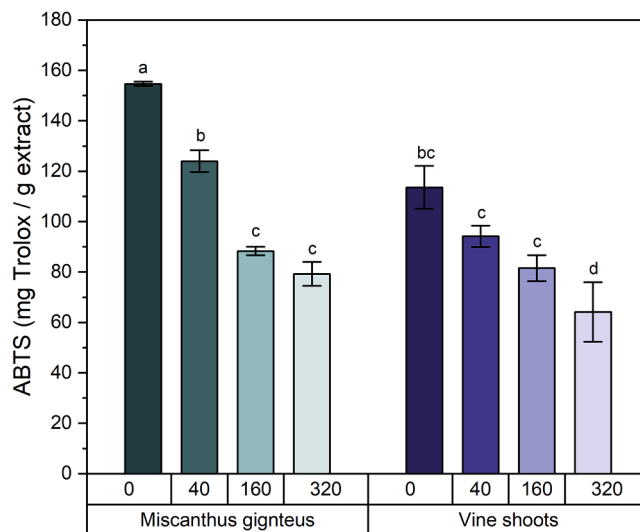


Fig. 3. ABTS antioxidant activity of water-soluble lignin extracts from ozonated *Miscanthus giganteus* and vine shoots at different ozonation times (0, 40, 160, 320 min). Different letters indicate significant differences between the mean values at $p < 0.05$ using Tukey-test.

ratio of lignin is widely associated with a stronger ROS scavenging ability (Cheng et al., 2020). Lignin extracts from *Miscanthus* might have a higher S/G ratio, potentially contributing to their higher scavenging ability compared with lignin extracts from vine shoots. Additionally, *Miscanthus* extracted fractions contained a higher quantity of phenolic monomers and a lower quantity of organic acids compared with vine shoots extracts (Table S1). Phenolic compounds exhibit higher antioxidant activity, whereas organic acids such as oxalic, tartaric, malic, and acetic acids are considered weak antioxidants. Notably, organic acids can inhibit the antioxidant activity of phenolic compounds (Zhang et al., 2019). Given the lower quantity of organic acids in *Miscanthus* extracts compared with vine shoots extracts, it is likely that organic acids had a less negative effect on their antioxidant activity.

Ozonation had a negative effect on the ABTS antioxidant activity of lignin extracted fractions, it resulted in a reduction of antioxidant activity, with the extracts obtained without ozonation showing the highest antioxidant activity: 154.6 and 113.6 mg Trolox/g extract for *Miscanthus* and vine shoots, respectively (Fig. 3). Despite the high ABTS antioxidant activity of these lignin extracts, their extraction yield was very low (2.6 % for *Miscanthus* and 4.3 % for vine shoots), rendering them economically unsuitable as antioxidants. Although extending the ozonation time reduced 48.7 % and 43.5 % of the ABTS antioxidant activity of *Miscanthus* and vine shoots lignin extracted fractions, respectively, the increase in the extraction yield was significantly higher (740 % and 485 % respectively) which could justify the use of ozonation to extract these antioxidants. However, ozonation increasingly generates carbonyl groups through oxidation, which has a negative effect on antioxidant activity. According to Ponomarenko et al. (2015), carbonyl groups in the side chain of the phenylpropane units reduce antioxidant activity, particularly in α position. This might be related to the electron-accepting properties of this group, which enhance the polarization of the O-H bond and makes its homolytic dissociation more difficult. If the carbonyl group is in other positions in the side chain, its negative effect on antioxidant activity is reduced (Ponomarenko et al., 2015). The generation of organic acids during ozonation may also have a negative effect on the antioxidant activity of phenolic compounds. There are other parameters such as the presence of non-etherified phenolic hydroxyl groups or aliphatic hydroxyl groups in the side chain, low lignin purity, and the increased size of conjugated system, all of which may negatively affect the antioxidant activity (Qin et al., 2020).

3.2.3. DPPH antioxidant activity

Fig. 4 illustrates the DPPH antioxidant activity of *Miscanthus* and vine shoots, represented as IC₅₀ values ($\mu\text{g}/\text{mL}$). Lower IC₅₀ values indicate more radical scavenging action, as it represents the concentration required for 50 % suppression of the DPPH radical. Ozonation increased the IC₅₀ values of vine shoots lignin extracts from 7.65 to 28.69 $\mu\text{g}/\text{mL}$, and *Miscanthus* lignin extracts from 17.41 to 27.48 $\mu\text{g}/\text{mL}$, compared with 9.23 $\mu\text{g}/\text{mL}$ for the Trolox standard. For comparison, Guo et al., (2020) reported an IC₅₀ of 0.042 mg/mL for lignin obtained using silicotungstic acids catalyzed ChCl/glycerol treatment of *Miscanthus giganteus*, which is higher than that of the ozonated *Miscanthus* lignin extracts. Phenolic-rich extracts from white wine grape pomace, obtained using subcritical water treatment, showed an IC₅₀ of 20.8 $\mu\text{g}/\text{mL}$ (Pedras et al., 2017), comparable to the ozonated vine shoots extracts. Other studies on corn straw lignin from enzymatic hydrolysis and steam explosion reported IC₅₀ values of 66 and 64 $\mu\text{g}/\text{mL}$, respectively, while BHT, another antioxidant standard, ranged from 53 to 56 $\mu\text{g}/\text{mL}$ (An et al., 2019, 2017). Arshanitsa et al. (2013) reported the IC₅₀ values for soluble lignin fractions from wheat straw ranging from 42 to 50 $\mu\text{g}/\text{mL}$. Lignin extracted from sugarcane bagasse using alkali and dilute acid alkali treatments showed IC₅₀ values of 243.5 and 38.5 $\mu\text{g}/\text{mL}$, respectively, in DPPH antioxidant assays (You et al., 2022).

The results demonstrate that lignin extracts from ozonated biomass exhibit strong DPPH antioxidant activity compared to other biomass-derived lignins, highlighting the potential of ozonation as a green

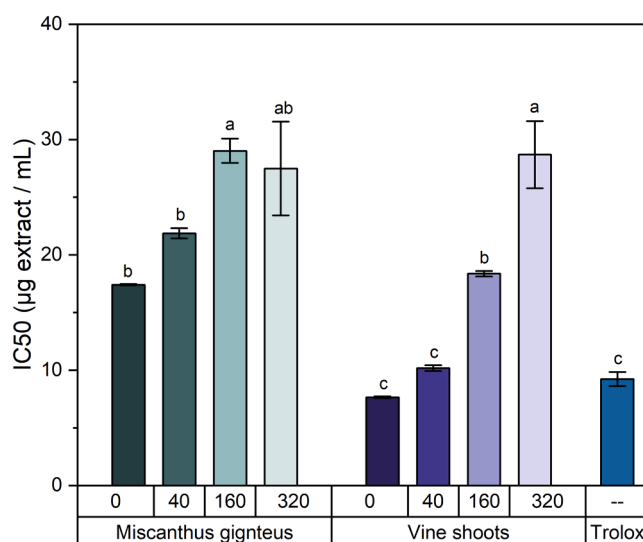


Fig. 4. DPPH antioxidant activity of water-soluble lignin extracts from ozonated *Miscanthus giganteus* and vine shoots at different ozonation times (0, 40, 160, 320 min). Different letters indicate significant differences between the mean values at $p < 0.05$ using Tukey-test.

strategy for the generation of lignin derived antioxidants. The structure of lignin, containing aromatic rings with hydroxyl and methoxyl functional groups, contributes to its antioxidant activity by donating hydrogen atoms and engaging in single electron transfer reaction (Alzameem et al., 2018). Additionally, the presence of phenolic acids enhances the antioxidant effect through resonance-stabilized phenoxyl radicals (Liu et al., 2020).

Similar to the ABTS results, ozonation decreased DPPH antioxidant activity. Shi et al. (2020) reported a similar result, with increase in IC₅₀ from 1 to 2 mg/mL for enzymatic lignin after ozonation. In contrast to ABTS antioxidant activity, vine shoots lignin extracts showed higher DPPH antioxidant activity compared with *Miscanthus* lignin extracts (Fig. 4), likely due to differences in radical scavenging mechanisms. Santos et al. (2014) reported a weak correlation between DPPH and ABTS antioxidant activity, due to the complexity of the reaction mechanism and the experimental sensitivity.

3.2.4. β -carotene bleaching

The β -carotene bleaching assay is an established technique for antioxidant activity analysis. It evaluates sample antioxidant efficacy by spectrophotometrically measuring changes in β -carotene concentration within β -carotene/peroxyl radicals (LOO \bullet) systems with and without antioxidants (Dawidowicz and Olszowy, 2010). This assay operates based on the hydrogen atom transfer (HAT) mechanism, measuring antioxidants' capacity to neutralize free radicals by donating hydrogen (Prior et al., 2005).

The results of the β -carotene bleaching assay for *Miscanthus* and vine shoots lignin extracts revealed superior antioxidant activity in *Miscanthus* extracts compared with vine shoots extracts (Fig. 5). *Miscanthus* lignin extracts maintained a high antioxidant activity until 160 min of ozonation, when yield increased, then declining to 45 % for the most ozonated fraction. The decline may be attributed to the degradation of antioxidant compounds operating via the HAT mechanism such as phenolic acids.

On the other hand, vine shoots lignin extracts showed different results. The inhibition of these extracts decreased from 70 % to 18 % for 0–40 min of ozonation (Fig. 5). Subsequently, the lignin extracts obtained at 160 and 320 min of ozonation exhibited a prooxidant effect in the β -carotene/peroxyl radical system, despite showing antioxidant activity in both ABTS and DPPH assays. This dual behavior aligns with findings that certain phenolic compounds can act as either antioxidant

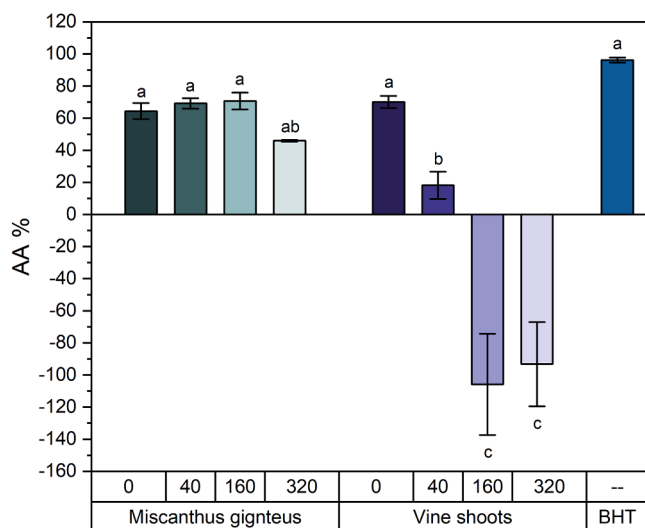


Fig. 5. β -carotene bleaching antioxidant activity (AA%) of water-soluble lignin extracts from ozonated *Miscanthus giganteus* and vine shoots at different ozonation times (0, 40, 160, 320 min). Different letters indicate significant differences between the mean values at $p < 0.05$ using Tukey-test.

or prooxidants depending on their concentration (Nowak et al., 2022). The solubility of these vine shoots lignin extracts in the lipid-based β -carotene system may differ from the more hydrophilic environment of the ABTS and DPPH assays, influencing their antioxidant and prooxidant activities. Additionally, the lignin extracts from these time points may function more as single electron donors (SET) rather than hydrogen atom donors (HAT), as evidenced by Folin-Ciocalteu results. Moreover, vine shoots released an insignificant quantity of phenolic acids during the ozonation process compared with *Miscanthus*, potentially contributing to the observed difference in β -carotene bleaching activity. Nevertheless, vine shoot lignin extracts displayed high ABTS and DPPH antioxidant activity, which rely on both SET and HAT mechanisms, suggesting that their ABTS and DPPH antioxidant activity may primarily result from single electron transfer.

To the best of our knowledge, β -carotene bleaching assays of technical lignin or lignin obtained through the ozonation process have not yet been reported. However, some research reported β -carotene results for phenols, with Ahmed et al. (2022) reported IC50 values for the β -carotene bleaching of extracts from *Carissa opaca* root, ranging from 2.79 to 4.71 mg/mL. Another study noted an 86.8 % β -carotene bleaching in extracts from *Lampaya medicinalis* Phil. (Verbenaceae) (Morales and Paredes, 2014). On the other hand, Gao et al. (2022) reported the β -carotene bleaching results for several phenolic acids, including 4-hydroxybenzoic acid, which demonstrated 22 % inhibition at a concentration of 500 μ g/mL. In brief, the high yield in these antioxidant extracts from relatively cheap residual biomass suggest they could be used for low-value food/feed applications, such as preservatives or packaging.

3.3. Antibacterial activity

The antibacterial activity of aqueous lignin extracted fractions from *Miscanthus* and vine shoots against *E. coli* and *S. aureus* was determined through *in vitro* assays using the broth microdilution method. Table 2 shows the MIC and MBC values of lignin extracted fractions, indicating that *Miscanthus* lignin extracted fractions exhibited greater antibacterial activity against both *E. coli* and *S. aureus* compared with vine shoot fractions. This difference may be related to differences in composition, including lignin oligomers, phenolic acids, phenolic aldehydes and organic acids, amongst others. *Miscanthus* extracted fractions contained lignin oligomers with lower molecular weight and polydispersity

Table 2

MIC and MBC of the aqueous lignin extracts of ozonated *Miscanthus giganteus* and vine shoots against the examined microorganisms.

	Ozonation time (min)	<i>S. aureus</i>		<i>E. coli</i>	
		MIC ^a (mg/mL)	MBC ^b (mg/mL)	MIC ^a (mg/mL)	MBC ^b (mg/mL)
<i>Miscanthus giganteus</i>	0	3	3	25	25
	40	3	6	6	6
	160	3	6	3	6
	320	3	6	6	6
Vine shoots	0	25	25	> 25	> 25
	40	25	> 25	> 25	> 25
	160	6	6	6	6
	320	3	6	6	6
Klason lignin sulfite	-	3	6	3	6
	-	1.5	1.5	1.5	3

^a MIC: minimum inhibitory concentration;

^b MBC: Minimum bactericidal concentration

compared with vine shoots (Table S1). The lignin oligomers with lower molecular weight may have better antibacterial activity because of higher penetration capacity through their cell walls (Li et al., 2023). Additionally, ozonation of *Miscanthus* generated higher quantities of phenolic monomers than vine shoots and phenolic acids such as 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid, which possess antibacterial activity (Neto et al., 2021). This difference in phenolic content may contribute to the higher antibacterial effectiveness observed in *Miscanthus* lignin extracts.

For vine shoot lignin extracts, MIC decreased from 25 to 3 mg/mL against *S. aureus* and from 25 to 6 mg/mL against *E. coli* by extending the ozonation time from 0 to 320 min, showing the effect of ozonation process on improving the antibacterial activity of lignin extracted fractions (Table 2). The antibacterial activity of lignin extracts, apart from being affected by composition, as previously mentioned, is also affected by the molecular weight of the lignin oligomers and/or the presence of carboxyl groups. Extending the ozonation time reduced the molecular weight of lignin oligomers in vine shoots extracts from 17,000 to 3600 g/mol and generated more homogeneous lignin oligomers (Table S1). Moreover, the extension of ozonation time generated a higher number of hydrophilic functional groups, which has been previously seen to reduce aggregation and enhance contact with the bacterial cells, thus causing extensive cell damage and death (Shirshahi et al., 2023). Lignin oligomers show antibacterial activity by direct contact with bacteria and rupture the cell membranes causing reactive oxygen species (ROS) to accumulate. The excess ROS is harmful to the cell's redox balance, and this imbalance results in oxidative stress, which changes the permeability of cell membrane and eventually causes damage and rupture (Li et al., 2023). The extension of the ozonation process has been reported to generate more carboxyl groups in the lignin extracted fractions (Ebrahimi et al., 2024) which will further decrease the pH level of the medium. Moreover, the generation of weak organic acids was sharply increased after 160 min of ozonation of vine shoots (according to GC-MS results from a previous article (Ebrahimi et al., 2024). Weak organic acids can act as antibacterials in their undissociated forms. These uncharged organic acid molecules can cause cellular harm in bacteria by penetrating their lipid membrane and entering their cytoplasm. The neutral intracellular pH level causes acid molecules to dissociate into charged protons and anions, which are subsequently unable to pass back through the plasma membrane. Generated H⁺-ions produce a reduction in intracellular pH to physiologically inappropriate levels, resulting in growth inhibition or cell death. This fundamental mode of action is often known as "weak organic acid theory" (Stratford et al., 2013). Therefore, the antibacterial activity of vine shoots lignin extracted fractions obtained at 160 and 320 min of ozonation is the result of different parameters such as lower molecular weight of lignin oligomers, higher quantity of carboxyl groups, and higher generation of

weak organic acids which improve the antibacterial activity. Comparing the MIC and MBC values of vine shoots extracts reveals that some extracts exhibit identical MIC and MBC values.

The bacterial growth curves of *E. coli* and *S. aureus* in the presence of vine shoots extracted fractions with different concentrations of 25, 12, 6, 3, 1.5, 0.6, and 0.4 mg/mL was studied by measuring the optical density value versus incubation time (Figure S1 & S2 in the Supplementary Material). As shown in these figures, extending the ozonation time reduced the bacterial growth of both *E. coli* and *S. aureus* compared with the lignin extract obtained without ozonation.

For *Miscanthus* lignin extracts, the results of MIC and MBC at different ozonation times showed that these fractions had better antibacterial activity against Gram-positive *S. aureus* than Gram-negative *E. coli*. However, the ozonation time did not affect the antibacterial activity of *Miscanthus* lignin extracts against *S. aureus*, while it had a positive effect on antibacterial activity against *E. coli*. MIC of *Miscanthus* lignin extracts from different ozonation times remained stable at 3 mg/mL against *S. aureus*, while it decreased from 25 to 3 mg/mL against *E. coli* by extending the ozonation time. The non-effect of ozonation time on the antibacterial activity against *S. aureus* could be related to the cell wall of this bacteria, which comprises a thin layer of peptidoglycan, teichoic acid, and numerous pores that allow foreign molecules to permeate (Wang et al., 2017). *Miscanthus* extracted fraction obtained without ozonation showed a MIC of 3 mg/mL against *S. aureus*, while the MIC was 25 mg/mL against *E. coli*. This fraction has lignin oligomers with a weight-average molecular weight of 11,000 g/mol, lower than vine shoot extracts, that might be able to penetrate the cell wall of *S. aureus* to a further extent than that of *E. coli*. Moreover, specific phenolic acids, such as 4-coumaric acid, have been reported to be present in *Miscanthus* lignin extracts and to exert antibacterial higher activity against *S. aureus* than against *E. coli* (Lou et al., 2012). By extending the ozonation time, the molecular weight of lignin oligomers in *Miscanthus* extracted fractions decreased, expecting to reduce the MIC against both bacteria as it was seen for vine shoot extracts. Although the effect of molecular weight was not strong enough to improve the antibacterial activity against *S. aureus*, this parameter had a positive effect on the MIC against *E. coli*, as the secondary cell wall of *E. coli* as a Gram-negative bacteria withstands the penetration of higher molecular weight lignin (Gil-Chávez et al., 2021). Therefore, the generation of carboxyl groups and weak organic acids by extending the ozonation time had a positive effect on the antibacterial activity of *Miscanthus* extracts against *E. coli*, while it did not have a significant effect on their antibacterial activity against *S. aureus*. The bacterial growth curves of *S. aureus* and *E. coli* in the presence of different concentrations (25, 12, 6, 3, 1.5, 0.6, and 0.4 mg/mL) of *Miscanthus* lignin extracts show that ozonation had a positive effect on the antibacterial activity of the extracts against both bacteria. However, the effect was not strong enough to decrease the minimum inhibitory concentration of the lignin extracts against *S. aureus* (Figures S3 & S4 in the Supplementary Material).

Comparing the antibacterial activity of *Miscanthus* and vine shoots lignin extracted fractions with commercial purified Kraft lignin, the most ozonated lignin extracted fractions exhibited similar antibacterial activity to that of commercial Kraft lignin, with a MIC of 3 mg/mL for both *E. coli* and *S. aureus*. The MIC of the high molecular weight technical lignin with M_w 4400–5000 g/mol against *E. coli* and *S. aureus* was reported to be 16 and 4 mg/mL, respectively (Sinisi et al., 2019). In another study, the antibacterial activity of bamboo kraft lignin after fractionation using ethanol with a molecular weight of 4030 g/mol was reported with a MIC value of 1 mg/mL against *S. aureus* and higher than 4 mg/mL against *E. coli* (Wang et al., 2019). The advantage of the lignin extracts obtained from ozonation lies in their environmentally-friendly extraction process without the use of hazardous chemical compounds. In addition, unlike commercial Kraft lignin, which is hydrophobic and insoluble in aqueous media, the lignin extracted fractions after ozonation are soluble in the medium, facilitating their application. The antibacterial activity of sulfite as an additive in wine was also investigated,

revealing a MIC of 1.5 mg/mL against *S. aureus* and *E. coli*, so the *Miscanthus* lignin extract at 160 min of ozonation and vine shoots lignin extracts at 320 min of ozonation with MIC of 3 mg/mL might potentially serve as an alternative for sulfite in wine application, amid others.

3.4. Antifungal analysis

The MIC of the lignin extracts against both fungi is presented in Table 3. *B. cinerea* showed more resistance to the lignin extracted fractions from both biomasses compared with *P. digitatum*. This difference might come from the plasma membrane H⁺-ATPase permeability between the two fungi (León Peláez et al., 2012). *Miscanthus* lignin extracted fractions showed better antifungal activity against both fungi compared with the lignin extracts obtained from vine shoots.

For *Miscanthus*, the extract obtained without ozonation did not show any antifungal activity against both fungi. The ozonation process improved the extraction of lignin fractions with higher antifungal activity. The lignin extracted fractions obtained after 40 and 160 min of ozonation of *Miscanthus* showed antifungal activity with a MIC of 12 mg/mL against *P. digitatum*. However, less inhibitory effect was observed for these extracts against *B. cinerea* where only lignin extracts after 160 min of ozonation inhibited the growth with 25 mg/mL concentration. For the lignin extracts obtained after 320 min of ozonation, a MIC of 25 mg/mL was observed for *P. digitatum* and *B. cinerea*. The ozonation process generates carbonyl and carboxyl groups in lignin extracted fractions, which might have a positive effect on the antifungal activity of the lignin extracts. Chitiva-Chitiva et al. (2021) reported that benzoic acid derivatives and dihydrochalcone demonstrated high antifungal activity against *Moniliophthora roreri* and *Fusarium solani* with benzoic acid derivatives having IC₅₀ of 9–27 µg/mL. Ando et al. (1986) reported the effect of different functional groups of phenolic compounds on their antifungal activity. Based on their results, the antifungal activity increased based on the contribution of different functional groups by this order: CH=CH > CHO > *p*-OH > COOH > *m*-OH > OCH₃. Therefore, the aldehyde groups have a stronger inhibition effect than carboxylic groups in aromatic compounds. For *Miscanthus* extracted fractions, 4-hydroxybenzaldehyde was higher at 40 and 160 min of ozonation, and then, by extending the ozonation time to 320 min, 4-hydroxybenzaldehyde sharply decreased, which might explain the increase in MIC of the *Miscanthus* extract against *P. digitatum* at 320 min of ozonation. However, for *B. cinerea*, the decrease in the aldehyde groups after 160 min did not affect the MIC. Another parameter that might affect this antifungal activity is the molecular weight of the lignin extracted fractions. The weight-average molecular weight of *Miscanthus* lignin extracted fractions at 40 and 160 min of ozonation was 6400 and 2600 g/mol which was much lower than 11,000 g/mol for the non-ozonated extract (Table S1). Ishimaru et al. (2023) reported that the *n*-hexane soluble fraction of organosolv lignin (10 wt%), with a low molecular weight of 542 g/mol, inhibited the growth of *Trametes*

Table 3
MIC of the aqueous lignin extracts of ozonated *Miscanthus giganteus* and vine shoots against the examined fungi.

	Ozonation time (min)	<i>P. digitatum</i>	<i>B. cinerea</i>
		MIC ^a (mg/mL)	MIC ^a (mg/mL)
<i>Miscanthus giganteus</i>	0	>25	>25
	40	12	>25
	160	12	25
	320	25	25
Vine shoots	0	>25	>25
	40	>25	>25
	160	>25	>25
	320	25	25
Sulfite	-	<0.4	<0.4

^a MIC: minimum inhibitory concentration

versicolor in a disc diffusion method. In contrast, the ethyl acetate soluble and insoluble fractions, with higher molecular weights of 1710 and 3894 g/mol, respectively, showed no antifungal activity.

For the vine shoots lignin extracts, no antifungal activity was observed for the extracted fractions obtained after 0, 40, and 160 min of ozonation against both *P. digitatum* and *B. cinerea*. The lignin extract obtained after 320 min of ozonation, with lower molecular weight (3600 g/mol), showed growth inhibition at 25 mg/mL against both fungi (Table 3). The lower antifungal activity of vine shoots fractions compared with *Miscanthus* fractions might come from their different structure. Vine shoots had higher molecular weight than *Miscanthus* lignin extracts and it has been observed that a lower molecular weight has a positive effect on antifungal activity. Moreover, phenolic monomers (phenolic acids and phenolic aldehydes) are in higher quantity in *Miscanthus* fractions than in vine shoots. Phenolic aldehydes and, to a lesser extent, phenolic acids, have positive effects on antifungal activity. Vine shoots lignin extracts have a higher quantity of organic acids than *Miscanthus* fractions. While organic acids had a positive effect on antibacterial activity, they were reported to not have a significant effect on antifungal activity (Hauser et al., 2016). The difference between the structure of bacteria and fungi might be one of the reasons. Fungi can be

single-cell or extremely complex multicellular eukaryotic organisms, whereas bacteria are unicellular prokaryote organisms lacking a nucleus and other membrane-bound organelles (Chen et al., 2023). Fungi can use the decarboxylation mechanism as a resistance to withstand the higher concentrations of organic acids to a further extent (Hauser et al., 2016). Sulfite, a common food preservative, showed high antifungal activity, with a MIC of less than 0.4 mg/mL against both fungi, in comparison to *Miscanthus* and vine shoots lignin extracts. This indicates that while *Miscanthus* and vine shoots lignin extracts exhibit good antibacterial activity relative to sulfite, they are less effective at inhibiting fungal growth. Further analysis should be done to a certain potential synergistic effect with sulfite or other preservatives.

Plotting the growth curves of both fungi in the presence of the lignin extracts posed a challenge due to a color change observed in the medium during the incubation period, which interrupted the optical density measurements. The color change might come from the interaction of the lignin extracts with the spores of the fungi in the medium. According to García et al. (2017), the growth and pigmentation of strains may be significantly impacted by the functionality and degree of substitution in the aromatic ring of phenolic compounds. Therefore, the microscopic pictures of the fungi cells were taken after 7 days of incubation to see the

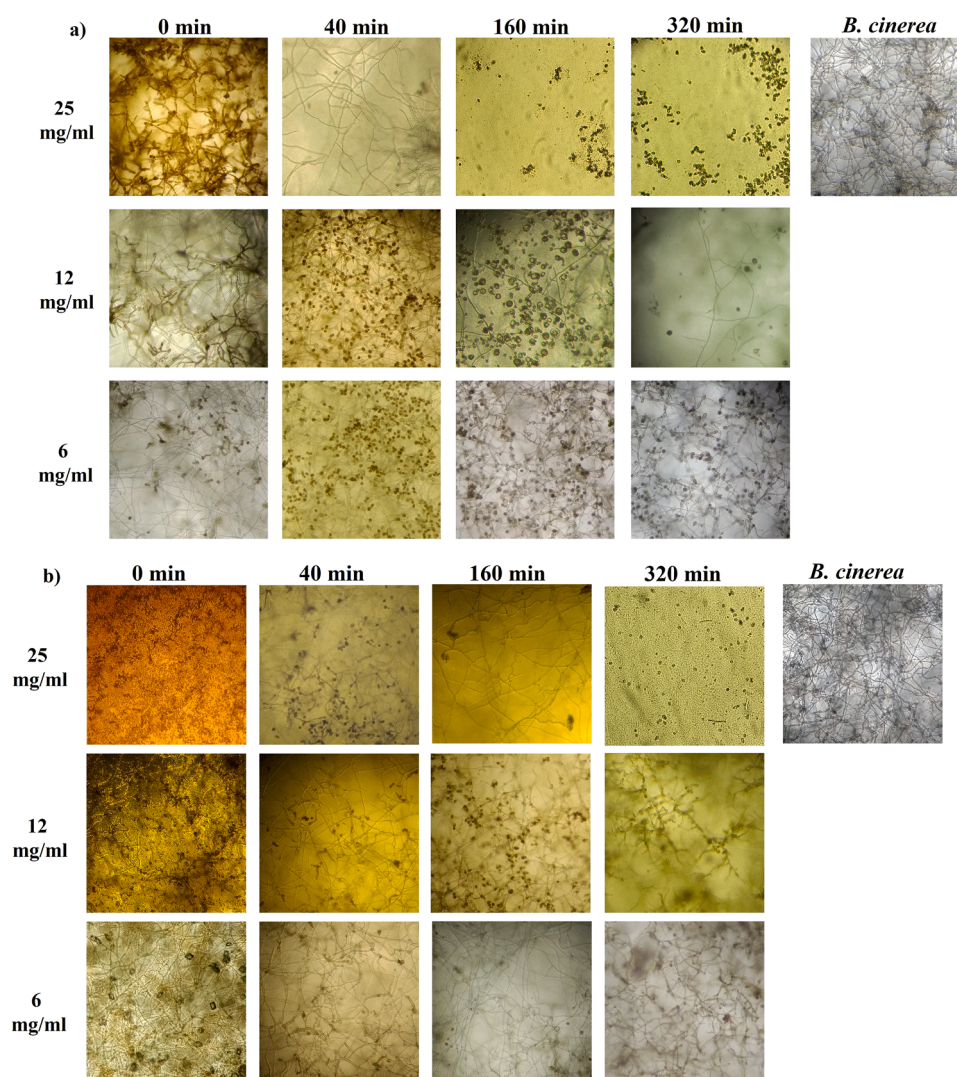


Fig. 6. Microscopic pictures of *B. cinerea* in the presence of water-soluble lignin extracts from ozonated a) *Miscanthus giganteus* and b) vine shoots after 7 days of incubation. The vertical values (25, 12, 6 mg/mL) represent different concentrations of lignin extracts from each biomass, and the horizontal values (0, 40, 160, 320 min) represent the extracts obtained at different time points of ozonation from each biomass. Also included a microscopic picture of *B. cinerea* without the extracts.

growth inhibition in the presence of lignin extracts.

The microscopic pictures with high concentrations of *Miscanthus* and vine shoot lignin extracts (25, 12, and 6 mg/mL) in the presence of *B. cinerea* after 7 days incubation are presented in Fig. 6. The growth inhibition can be observed in microscopic pictures and it is in line with the results observed in 96-well plates. For *Miscanthus* extracted fractions, no growth of *B. cinerea* was observed for the lignin extracts at 160 and 320 min of ozonation with 25 mg/mL, and the extract at 320 min of ozonation with 12 mg/mL reduced the growth of *B. cinerea* (Fig. 6a). For vine shoots, only the lignin extracts after 320 min of ozonation inhibited the growth of *B. cinerea* at 25 mg/mL and reduced the fungi growth with

12 mg/mL concentration (Fig. 6b). The microscopic pictures of *Miscanthus* and vine shoot lignin extracts (25, 12, 6, and 0.4 mg/mL) in the presence of *P. digitatum* after 7 days of incubation are presented in Fig. 7. The growth inhibition of the fungi was observed only for the extracts obtained after the ozonation process. For *Miscanthus*, the lignin extracts from 40 and 160 min of ozonation showed a MIC of 12 mg/mL, while the one from 320 min of ozonation showed a MIC of 25 mg/mL (Fig. 7a). For vine shoots, only the lignin extract after 320 min of ozonation showed a MIC value of 25 mg/mL (Fig. 7b).

The antifungal activity of lignin has been scarcely studied. Sinisi et al. (2019) reported that a technical lignin with high molecular weight

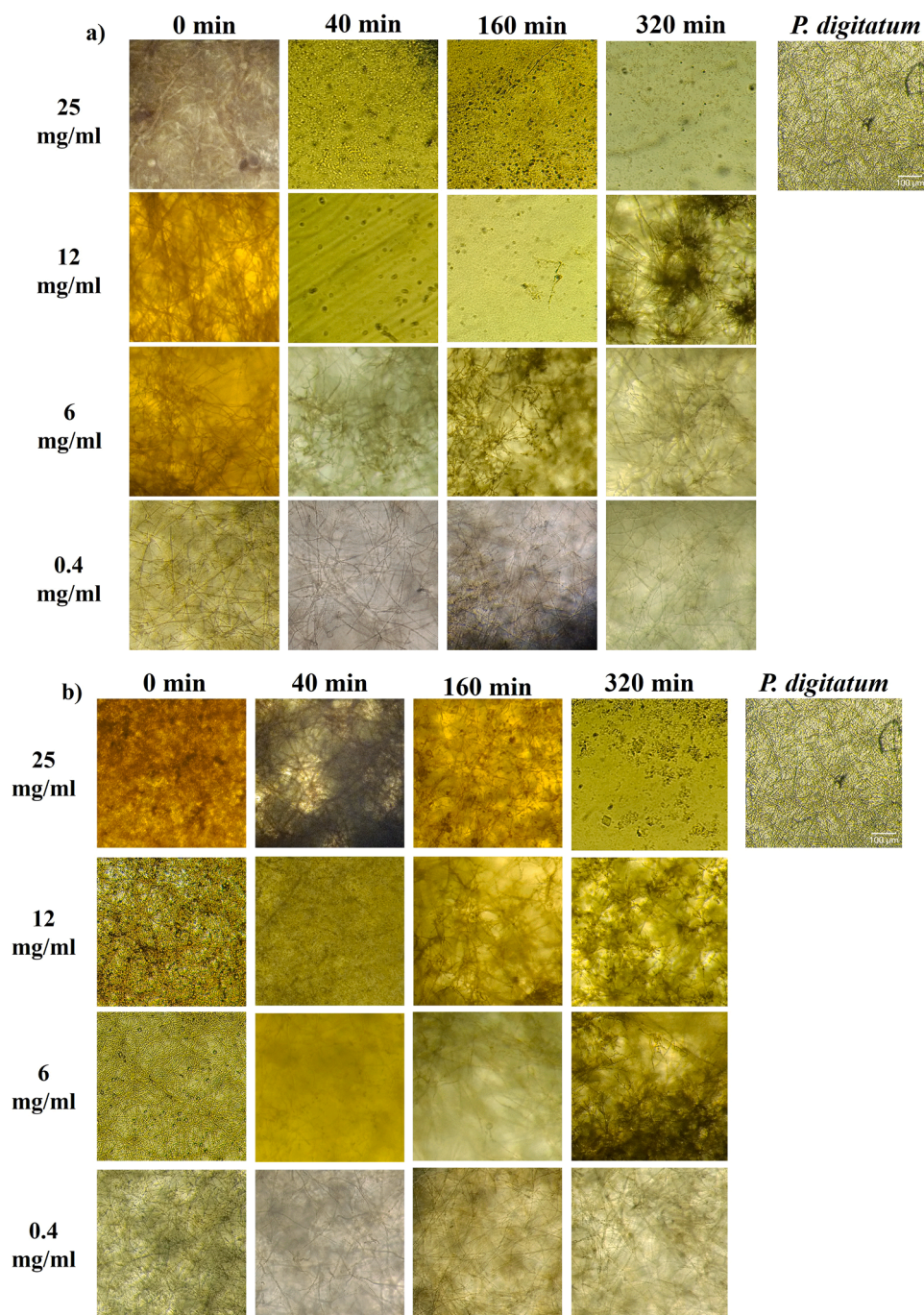


Fig. 7. Microscopic pictures of *P. digitatum* in the presence of water-soluble lignin extracts from ozonated a) *Miscanthus giganteus* and b) vine shoots after 7 days of incubation. The vertical values (25, 12, 6, 0.4 mg/mL) represent different concentrations of lignin extracts from each biomass, and the horizontal values (0, 40, 160, 320 min) represent the extracts obtained at different time points of ozonation from each biomass. Also included a microscopic picture of *P. digitatum* without the extracts.

from 4400 – 5000 g/mol did not show any antifungal activity against *B. cinerea* (MIC > 32 mg/mL). Núñez-Flores et al. (2012) did not observe any antifungal activity of lignosulfonate against *Penicillium expansum*. Lignin extracts from corn stover showed no fungicidal action with a maximum concentration of 10 mg/mL (Dong et al., 2011). In another study, the Kraft lignin did not show any antifungal activity against *B. cinerea* (MIC > 50 mg/mL) (Gazzurelli et al., 2022). In this study, *Miscanthus* and vine shoots lignin extracts obtained after ozonation showed better antifungal activity than other technical lignins, likely due to the carboxylic and aldehyde groups generated in the aromatic compounds in lignin oligomers and phenolic monomers. The increased solubility of lignin extracts, resulting from the hydrophilic functional groups formed during ozonation, may also contribute to their antifungal activity. However, despite better MIC values compared with technical lignins, the values are still high for the extracts to be considered biologically applicable as antifungal agents.

3.5. Zeta potential analysis

Zeta potential analysis was performed on lignin extracted fractions to evaluate its impact on their antimicrobial activity, with results detailed in Table 4. Lignin extracts from *Miscanthus* exhibited predominantly positive zeta potential values, while vine shoots lignin extracts, except for the one obtained after 40 min of ozonation, exhibited a positive near-zero surface charge. *E. coli* and *S. aureus* were reported to possess negative charges, with zeta potential values of -22.6 and -30.9 , respectively (Andrade et al., 2015). Bacteria with a negative charge have a stronger preference for materials with a positive or nearly neutral surface charge than those with a negative charge (Li et al., 2023). Therefore, the positive charge present in *Miscanthus* extracts could influence their affinity towards both bacteria and their antimicrobial efficacy, whereas vine shoots with a near zero surface charge may exhibit affinity to bacteria, resulting in reduced antimicrobial activity. Furthermore, the higher surface charge of *S. aureus* compared with *E. coli* could contribute to the superior antimicrobial activity against this bacterium.

4. Conclusion

Water soluble lignin extracts with significant bioactive properties were obtained through ozonation of *Miscanthus giganteus* and vine shoots. The total polyphenol yield per gram of biomass was much enhanced through ozonation time, although the TPC and antioxidant activity per gram of lignin extract decreased. *Miscanthus* extracts could potentially employ both SET and HAT mechanisms, while vine shoots may primarily utilize the SET mechanism. The β -carotene bleaching assay confirmed the strong antioxidant potential of *Miscanthus* extracts. Ozonation enhanced the antimicrobial activity of the lignin extracts, with *Miscanthus* showing superior efficiency. A correlation was observed between the zeta potential of the extracts and their antimicrobial properties. The combination of ozonation and water extraction proved to be an environmentally friendly and promising approach for producing water soluble lignin fractions with valuable bioactive properties, which have potential applications in fields such as food and food packaging.

CRediT authorship contribution statement

Mahrokh Ebrahimi: Writing – original draft, Investigation, Formal analysis, Data curation. **Amparo López-Rubio:** Writing – review & editing, Validation, Conceptualization. **Ana-Rosa Ballester:** Writing – review & editing, Supervision, Resources, Methodology. **Victor Acha:** Writing – review & editing, Validation, Conceptualization. **Antonio Martínez-Abad:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Thierry Aussenac:** Project administration, Funding acquisition.

Table 4

Zeta potential of water-soluble lignin extracts from ozonated *Miscanthus giganteus* and vine shoots at different ozonation times.

Ozonation time (min)	Zeta potential (mV)	
	<i>Miscanthus giganteus</i>	Vine shoots
0	$14.0^a \pm 0.31$	$3.1^a \pm 0.10$
40	$13.1^a \pm 0.21$	$-4.4^b \pm 1.87$
160	$12.4^a \pm 0.70$	$0.2^{ab} \pm 0.56$
320	$15.4^a \pm 0.44$	$1.7^a \pm 0.59$

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.indcrop.2024.120092](https://doi.org/10.1016/j.indcrop.2024.120092).

Data availability

Data will be made available on request.

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