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CHEMICAL SCIENCES

Alkaline lignins from *Morinda citrifolia* leaves are potential immunomodulatory, antitumor, and antimicrobial agents

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Abstract: *Morinda citrifolia*, commonly known as noni, is a plant belonging to the Rubiaceae family. This plant has a high biological potential, which has different biological properties, including antioxidant, antibacterial, antiviral, antifungal, antitumor and anti-inflammatory. In this work, the immunomodulatory, antitumor and antimicrobial activities of lignin isolated from *Morinda citrifolia* leaves were investigated. The results showed that this lignin was not cytotoxic and that it was able to promote activation and differentiation of immune cells in addition to inducing the production of anti-inflammatory cytokines. Furthermore, it was able to inhibit the growth of different tumor and microbial cells *in vitro*. This pioneering study on these different activities shows that the lignin isolated in this study can be used as a raw material to obtain biomedical and pharmaceutical products.

Key words: Biological activities, lignin, macromolecules, pharmaceutical applications.

INTRODUCTION

Lignin is a macromolecule derived from phenylpropanoid units called C_6C_3, or C_9 units, irregularly repeated (Huang et al. 2022). Present in different lignocellulosic biomasses, its biosynthesis takes place via the root route from the reaction of three different precursor cinnamyl alcohols: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol, which generate guaicyl (G), syringyl (S) and *p*-hydroxybenzyl units (H), respectively (Huang et al. 2022, Sugiarto et al. 2022). The proportion of these components may vary between species (Sethupathy et al. 2022). Knowing the main constituents, functional groups and chemical bonds present in the macromolecular structure of lignin is extremely important for possible applications (Sugiarto et al. 2022).

Routinely, lignins are obtained on an industrial scale as a by-product of the paper industry, along with cellulose, which in turn is burned for energy production (Zhang et al. 2022). However, when recovered, they can be used as raw material to obtain products with high added value (Agustiany et al. 2022). The literature has presented several applications for lignins, among them applications related to biomedical areas (Sugiarto et al. 2022), such as antioxidant, antitumor (Araujo et al. 2022), immunostimulant (Cruz Filho et al. 2019, Nascimento Santos et al. 2020), antiparasitic (Silva et al. 2021), antiviral (Kimura et al. 2022) among others. And all these applications are directly related to the chemical structure (Kimura et al. 2022, Sugiarto et al. 2022).

Therefore, there is a growing interest in the study and probable applications of lignins both *in natura* and their fractionation products

obtained from different plants and/or processes (Naim et al. 2022).

Given this diversity of plant species, we highlight here the species *Morinda citrifolia* popularly known as Noni. This species has been widely used for medicinal purposes (Oladeji et al. 2022). Even though much is known about the extracts of this plant, little is known about the lignins of this species. Pioneering work carried out in our research group and published by Silva et al. (2021) addressed the physicochemical characterization of alkaline lignin obtained from the leaves of *Morinda citrifolia*, in addition to verifying that these can act as a promising antioxidant and antiparasitic agent.

Therefore, the present work continues the work proposed by Silva et al (2021) that is, it addresses in an unprecedented way other biological applications for lignin from the leaves of *Morinda citrifolia*, showing that these can act as an immunomodulatory, antitumor and antimicrobial agent.

MATERIALS AND METHODS

Lignin from the leaves of *Morinda citrifolia citrifolia*

Morinda citrifolia leaves were collected in the municipality of Cabo de Santo Agostinho located in the state of Pernambuco, Brazil (8°29'86.07" S and 35°06'45.29" W). Botanical authentication was carried out at the Herbarium Geraldo Mariz, at the Botany Department of the Biological Sciences Center of the Federal University of Pernambuco, where a specimen was deposited under registration number 74,792. To authorize the collection and study of the stem, the species was registered in SisGen (National System of Genetic Heritage and Associated Traditional Knowledge), No. A2F0474.

The alkaline lignin evaluated in this study was isolated from *Morinda citrifolia* leaves at the Laboratory of Chemistry and Therapeutic Innovation at the Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil, and published by Silva et al. (2021). Briefly, the leaves of *Morinda citrifolia* were dried and ground, then subjected to an aqueous extraction, in the proportion (g/L) 1:1, in a 2 L reactor, at 60 °C and 1200 rpm, for 1 h. The solid obtained in the extraction step was subjected to an acid treatment with H_3PO_4 (1%) in an autoclave at 121 °C for 1 h, 1:1 solid: liquid ratio (g/L). The hydrolyzed acid is filtered and the resulting solid was subjected to alkaline delignification with 1% NaOH under the same conditions as in the acid treatment. After the end of the reaction, the black liquor containing the lignin was separated from the resulting solid by filtration and soon after it was acidified with H_2SO_4 at pH 2 for 12 h to precipitate the lignin. Finally, the lignin obtained was dried at 70 °C for 48 h. Therefore, to carry out the biological assays, the lignin was previously diluted in 1% DMSO at a concentration of 200 µg/mL.

In vitro immunomodulatory activity

Animals, ethical considerations, and obtaining splenocytes

Splenocytes were obtained according to the methodology proposed by Cruz Filho et al. (2019). Female Balb/c mice (6 to 8 weeks old and 5 animals) were maintained under standard laboratory conditions (20 to 22 °C and 12 h day and night cycle) with free access to water and standard diet in Keizo Asami's vivarium Laboratory of Immunopathology (LIKA), located at the Federal University of Pernambuco, Recife, Pernambuco, Brazil. The animals were anesthetized with 2% xylazine (10 mg/Kg) and 10% ketamine hydrochloride (115 mg/Kg) and euthanized by cervical dislocation.

Spleens were aseptically removed and placed in culture tubes containing RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) with fetal bovine serum. Splenocytes were obtained by separation in Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Sweden) with density adjusted to 1.077 g/mL. Cell viability (> 98%) was assessed by the trypan blue exclusion method. This study was approved by the Committee on Ethics in the Use of Animals of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz, protocol number 164/2020.

Evaluation of splenocyte viability and proliferation

Viability assessment against splenocytes (1x 10^6 cells/well) was performed according to Cruz Filho et al. (2019) in different concentrations of lignins (100 to 3.12 µg/mL diluted in 1% DMSO) for 24 h in a CO_2 oven. After that, the cells were centrifuged, stained with propidium iodide and annexin V for 10 min and analyzed in a flow cytometer (FACS Calibur platform) in 10000 events. Data were analyzed in Flowing 2.0.1 software®. Cell proliferation assays were performed with CFSE (Carboxyfluorescein succinimidyl ester) under the same experimental conditions as the viability assays. All experiments were performed in quintuplicate.

Determination of cytosolic and mitochondrial levels of reactive oxygen species, calcium release, and changes in membrane potential

The experiments were performed according to Cruz Filho et al. (2019) and Nascimento Santos et al. (2020) with few modifications. Cytosolic and mitochondrial levels of reactive oxygen species (ROS), determination of transmembrane potential, and cytosolic Ca^{2+} concentrations in cells (1x 10^6 cells/well) treated with lignin (12.5 µg/mL) were determined by flow cytometry using dihydroethidium (DHE) (Merck), MitoSox Red (Thermo Fischer Fisher-USA), MitoStatus (BD Biosciences-USA) and fluo-3AM (Thermo Fisher Scientific-USA), respectively. All experiments were performed in quintuplicate.

Immunophenotyping assays

The immunophenotyping assay of lymphocytes and monocytes was performed according to Nascimento Santos et al. (2020) with modifications. Splenocytes were also stimulated with 12.5 µg/mL lignin. The positive control was cells only. The system was incubated for 24 h in RPMI 1640 medium at 5% CO_2. The mouse monoclonal antibodies used were anti-CD4-FITC, anti-CD8-PE for lymphocytes, anti-CD16-PE for natural killer cells and anti-CD16/32-PercyP for monocytes. All results were acquired in a flow cytometer using the FACS Calibur platform and the results were analyzed using the Flowing 2.5.1 ® software and the graphs were plotted using the Prism 6.0 ® software. All experiments were performed in quintuplicate.

Investigation of cytokine and nitric oxide production

The investigation of cytokine and nitric oxide production was performed according to Cruz Filho et al. (2019) and Nascimento Santos et al. (2020). From the supernatant obtained in the cell viability assays, cytokine levels were determined using the mouse cytokine kit (CBA). Cytokines evaluated were IL-2, -4, -6, -10, -17 TNF-α and IFN-γ. All data were acquired in flow cytometry using the FACS Calibur platform (BD®) and the results were analyzed using the Flowing 2.5.1® Software. Nitric oxide released by the same cells was determined by the Griess method, using a sodium nitrite standard curve (3.12-400 µM). Absorbances were determined in a microplate spectrophotometer (Multiskan FC, Thermo Scientific®) at 595 nm. The experiments were performed in triplicate.

Cytotoxicity assays against normal and tumor cell lines

The *in vitro* cytotoxicity experiments were performed according to the methodology proposed by Suleiman & Helal (2022). Cultures of normal (macrophages (RAW 264.7), fibroblasts (V79) and vero cells) and tumor (MCF-7 (breast cancer), T-47D (breast cancer), Jurkat (leukemia/lymphoma), DU145 (breast cancer) cell lines of human prostate cancer) and HepG2 (hepatoma)) were cultured in RPMI medium with phenol red supplemented with 10% FBS and 1% antibiotic, at a concentration of 1×10^6 cells/well were treated with lignin (dissolved in DMSO 1%) in concentrations ranging from 100 to 1.56 µg/mL for 72 h in a CO_2 oven and the cytotoxicity was determined by the MTT assay. After cultivation, the formazan crystals were solubilized in DMSO and the absorbance was evaluated in a plate reader at 540 nm. The 50% growth inhibition concentration (IC_{50}) was determined by non-linear fit. Furthermore, the selectivity index (SI) was determined, calculated as the ratio of normal cell IC_{50} to tumor cell IC_{50}. The patterns evaluated against tumor cells were: Doxorubicin, Amsacrine and Asulacrine evaluated under the same experimental conditions as lignin. All experiments were performed in two replicates for each of three independent experiments.

In vitro hemolytic activity

The hemolytic assay, used to assess in vitro toxicity, was performed by diluting erythrocytes from mouse blood in phosphate saline buffer that received different concentrations of lignin (100 to 3.12 µg/mL diluted in 1% DMSO). After 1 h, the release of hemoglobin (hemolysis) was evaluated by spectrophotometry. A saline solution was the negative control 0% hemolysis and triton X was the positive control 100% hemolysis. All experiments were performed in triplicate (Araujo et al. 2022).

In vitro antimicrobial activity

The antimicrobial activity experiments were carried out according to the methodology proposed by Suleiman & Helal (2022), Suleiman et al. (2022) and Suleiman (2020) with few modifications. For this, bacterial strains (*Acinetobacter baumannii* UFPEDA-1024, *Enterococcus faecalis* UFPEDA-138, *Enterococcus faecalis* UFPEDA-69, *Staphylococcus aureus* UFPEDA-709, *Pseudomonas aeruginosa* UFPEDA-416, *Pseudomonas aeruginosa* UFPEDA-261) and yeast strains (*Candida albicans* URM 95, *Candida albicans* 4664, *Candida albicans* UFPEDA-1007, *Candida guilliermondii* UFPEDA-6390 and *Candida glabrata* UFPEDA-6393). The standardization of the inoculum (bacteria and yeasts) was performed following the documents of the Clinical Laboratory Standards Institute (CLSI), M100 (CLSI 2017) and M60 (CLSI 2018), respectively.

The determination of the minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC). Serial dilutions were performed in DMSO 1% of the lignins and standards (Gentamicin, Amikacin, Oxacillin and Ampicillin+Sulbactam, against bacteria and Micafungin for yeasts), varying the concentration from 1024 to 4 µg/mL. Assays were performed in 96-well plates incubated for 24 h at 37 °C (bacteria) and 30 °C for 48 h (yeasts). The 0.01% resazurin solution was used as an indicator of bacterial growth. Cultures were plated on Mueller Hinton (Bacteria) or Sabourand Agar (Yeast) medium to determine MBC/MFC. Activity against the microorganism was considered when the MIC was ≤ 1024 µg/mL. The experiments were performed in triplicate.

Statistical analysis

Results were expressed as mean ± standard deviation and were treated with a one-way ANOVA statistical test for comparison between

groups and differences assessed by Tukey's post-test ($p < 0.05$).

RESULTS AND DISCUSSION

Evaluation of *in vitro* immunomodulatory activity

Figure 1 shows the viability results by the propidium iodide and annexin V method (Figure 1a) and cell proliferation (Figure 1b).

The results showed that the evaluated lignin was not able to promote cell death of splenocytes, promoting cell viability greater than 95%, moreover, it was able to promote cell proliferation in the different concentrations evaluated. Similar results were obtained by Cruz Filho et al. (2019), Melo et al. (2020), Nascimento Santos et al. (2020), and Araújo et al. (2022) evaluating the effect of different alkaline lignins against immune cells. As none of the evaluated lignin concentrations were cytotoxic, we chose the concentration of 12.5 µg/mL, a concentration routinely used for the evaluation of cytokines and nitric oxide promoted by different lignins. The concentration of cytokines and nitric oxide in the supernatant is shown in Table I.

The results showed that lignin induced an increase in most anti-inflammatory cytokines, that is, of the Th2 profile, such as IL-4, IL-10, and IL-6 cytokines, the latter being of a pleiotropic nature. In addition, an increase in the production of IL-2 was also observed. Confirmation of the anti-inflammatory profile promoted by lignin is in the results of the inhibition of nitric oxide production by cells treated with lignin at a concentration of 12.5 µg/mL.

Similar results were obtained by Araujo et al. (2022), Melo et al. (2020), and Arruda et al. (2022) also evaluating alkaline lignins. However, Cruz Filho et al. (2019) and Nascimento Santos et al. (2020) obtained a pro-inflammatory response for their evaluated alkaline lignins. The mechanism of the immunomodulatory activity promoted by lignins is not fully understood, however, it is known that the differences in the immune response promoted by lignins may be associated with the complex and variable chemical structure of these macromolecules (Cruz Filho et al. 2019, Nascimento Santos et al. 2020). This is

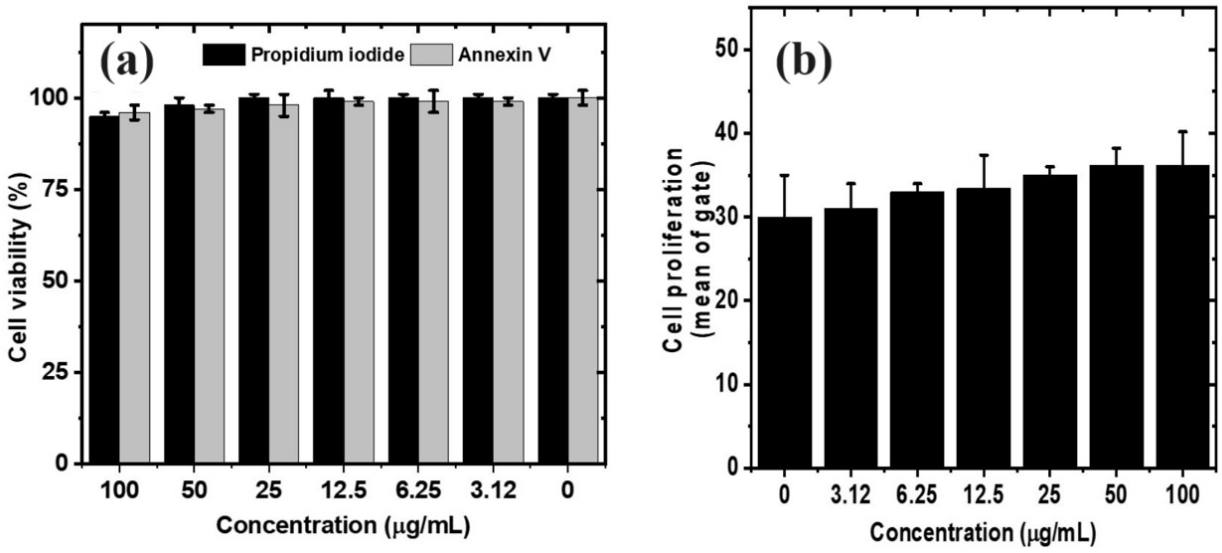

Figure 1. Results of viability promoted by lignin against splenocyte cells evaluated by staining with propidium iodide and annexin (a) and cell proliferation (b) respectively.

Table I. Production of cytokines and nitric oxide stimulated by alkaline lignin from *Morinda citrifolia* at a concentration of 12.5 µg /mL for 24 hours of incubation of splenocytes.

Cytokines (pg/mL)	Control	Lignin
IL-2	9.45 ± 0.1	10.59 ± 0.5
IL-4	8.97 ± 0.9	10.24 ± 0.8
IL-6	10.98 ± 0.1	12.76 ± 0.2
IL-10	11.91 ± 0.2	13.98 ± 0.0
IL-17	6.81 ± 0.1	6.84 ± 0.9
TNF-α	5.98 ± 0.5	5.92 ± 0.1
IFN-γ	8.34 ± 0.0	8.30 ± 0.1
Nitric oxide (µg/mL)	0.09 ± 0.0	0.03± 0.0

Mean ± Standard deviation.

because different functional groups present in the structure can be recognized by one or more receptors present on the cell surface, which can promote different immune responses *in vitro* (Arruda et al. 2021).

In addition to cytotoxins, cytosolic and mitochondrial levels of reactive oxygen species (ROS), calcium release, and changes in membrane potential were determined at a concentration of 12.5 µg/mL of lignin, and the results are shown in Figure 2.

The results presented in Figure 2 showed that lignin (12.5 µg/mL) was able to promote a non-significant increase in both cytosolic (Figure 2a) and mitochondrial (Figure 2b) ROS production and in cytosolic calcium levels (Figure 2c). However, a decrease in mitochondrial membrane potential was observed (Figure 2d). The response to oxidative stress, without inducing cell death, is an important step when it comes to the differentiation and activation of immune cells (Nascimento Santos et al. 2020). Similar results were obtained by Cruz Filho et al. (2019), Melo et al. (2020), and Nascimento Santos et al. (2020) evaluating different alkaline lignins against immune cells.

Finally, the immunophenotyping assay of lymphocytes and monocytes showed that lignin promoted the proliferation and activation of CD8+T, with no significant difference in the activation of CD4+ and C16+T lymphocytes. Regarding monocytes, there was no stimulus for differentiation. An increased population of CD8+T lymphocytes plays an important role in the immune response against tumors. Therefore, several antitumor strategies have been developed using molecules capable of promoting increased expression of CD8+T cells. Similar results were obtained by Nascimento Santos et al. (2020) evaluating alkaline lignins obtained from leaves. Therefore, the results presented here show that lignins, in addition to not being cytotoxic, can promote cell proliferation, induce the production of anti-inflammatory cytokines and promote the activation of immune cells.

Cytotoxicity assays against normal and tumor cell lines and *in vitro* hemolytic activity

Lignins are described in the literature as having antitumor activity *in vitro* and this is due to their phenolic nature. Table II presents only the results of cytotoxicity promoted by the lignin under study against tumor cells. Since it was not possible to determine the IC_{50} (concentration that inhibits cell growth by 50% against normal cells) in the evaluated concentrations. Furthermore, lignins were not hemolytic (~5%).

The results show that lignin presented IC_{50} values for all evaluated cells. Lower IC_{50} values represent higher inhibition values, so we can group the results in the following order: Jurkat > MCF-7 > T47D > DU145 > HepG2. However, when compared to the evaluated standards, lignin showed a low inhibition result, that is, higher IC_{50} values. As the lignins were not cytotoxic against normal cells, it was not possible to determine the selectivity index. Therefore, we can say that

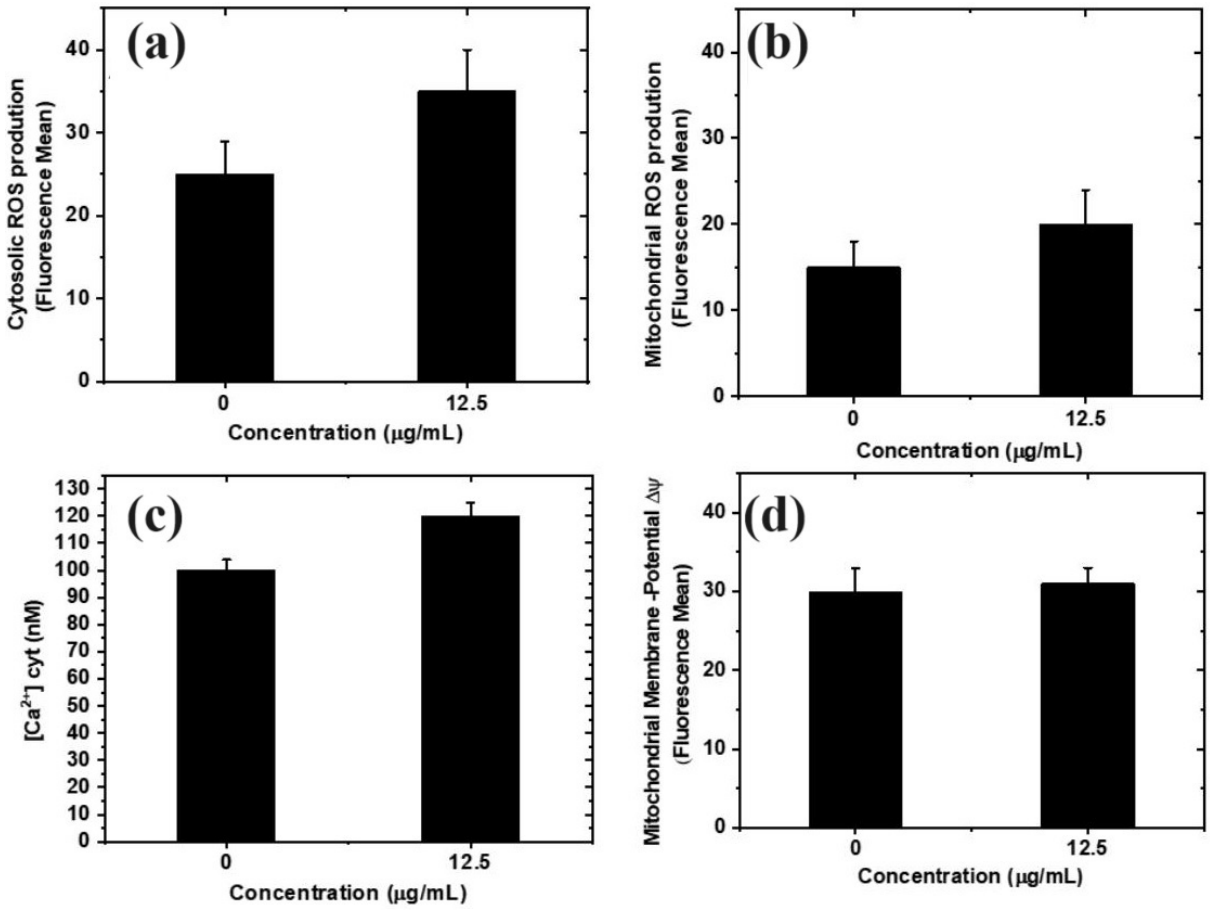

Figure 2. Cytosolic (a) and mitochondrial (b) levels of reactive oxygen species (ROS), calcium release (c) and mitochondrial membrane potential (ΔΨ) (d) respectively.

the lignin under study is cytotoxic only for the evaluated tumor cells.

Results close to ours were obtained by Araujo et al. (2022) evaluating alkaline lignins obtained from the branches and leaves of *Buchenavia viridiflora* respectively obtained IC_{50} values for the cells: Jurkat (21.37; 25.76 µg/mL), MCF-7 (12.63; 24.88 µg/mL), T47D (25.46; 47.74 µg/mL), DU145 (37.56; 41.65 µg/mL) and HepG2 (>100 µg/mL). Barapatre et al. (2016) evaluating four fractions of lignin obtained from *Acacia nilotica*, found that they had a higher cytotoxic potential (IC_{50}: 2–15 µg/mL) for the breast cancer cell line (MCF-7), but were ineffective (IC_{50}: ≥ 100 µg/mL) against normal primary human hepatic stellate cells (HHSteCs).

These findings indicate that the lignin under study has *in vitro* antitumor potential, corroborating the results obtained in immunomodulatory activity, where it was possible to verify the production of anti-inflammatory cytokines and a greater number of CD8+T lymphocytes in the population, important cells for inhibiting the growth of tumors.

In vitro antimicrobial activity

Lignins are described as promising antimicrobial agents (Melo et al. 2020, Araujo et al. 2022). Table III presents the results of *in vitro* antimicrobial activity promoted by lignin from *Morinda citrifolia* leaves against different species of bacteria and fungi.

Table II. ***In vitro*** **antitumor activity assays promoted by lignins compared to different standards.**

	Lignin	Doxorubicin	Amsacrine	Asulacrine
Tumor cells	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**	**IC_{50} (µg/mL)**
MCF-7	27.0 ± 0.1	1.0 ± 0.3	1.3 ± 0.1	1.2 ± 0.1
T-47D	32.7 ± 0.4	1.3 ± 0.1	1.2 ± 0.4	1.5 ± 0.4
Jurkat	19.4 ± 0.2	0.7 ± 0.0	1.4 ± 0.1	1.3 ± 0.1
DU145	52.3 ± 0.9	0.76 ± 0.0	0.80 ± 0.01	1.37± 0.1
HepG2	75.6 ± 0.1	1.54± 0.1	0.66 ± 0.0	1.4 ± 0.1

Mean ± Standard deviation.

Table III. Results of ***in vitro*** **antimicrobial activity promoted by lignin from** ***Morinda citrifolia*** **leaves.**

Microorganisms	**Lignin**		**Control - antimicrobial**	
Bacteria	**MIC (µg/mL)**	**MBC (µg/mL)**	**MIC** (µg/mL)	
Enterococcus faecalis UFPEDA-69	> 1024	> 1024	Amikacin	8 (S)
Enterococcus faecalis UFPEDA-138	>1024	> 1024	Amikacin	8 (S)
Staphylococcus aureus UFPEDA-709	512	> 1024	Oxacillin	512 (R)
Acinetobacter baumannii UFPEDA-1024	> 1024	> 1024	Amp.+Sulbac.	16 (I)
Pseudomonas aeruginosa UFPEDA-261	> 1024	> 1024	Amikacin	32 (I)
Pseudomonas aeruginosa UFPEDA-416	> 1024	> 1024	Amikacin	8 (S)
Yeasts	**MIC (µg/mL)**	**MFC (µg/mL)**	**MIC** (µg/mL)	
Candida albicans UFPEDA-1007	> 1024	> 1024	Micafungin	0.0625 (S)
Candida albicans 95	> 1024	> 1024	Micafungin	0.0312 (S)
Candida albicans 4664	512	> 1024	Micafungin	0.0156 (S)
Candida glabrata UFPEDA-6393	512	> 1024	Micafungin	0.0156 (S)
Candida guilliermondii UFPEDA-6390	> 1024	> 1024	Micafungin	0.0156 (S)

MIC – Minimum inhibitory concentration; MBC – Minimum bactericidal concentration; MFC – Minimum fungicidal concentration; R – Resistant; S – Sensitive; I – Intermediate resistance; Amp.+Sulbac – Ampicillin+Sulbactam.

The results presented in Table III showed that the evaluated lignin was capable of promoting antimicrobial activity, presenting a minimum inhibitory concentration of 512 µg/mL for *Staphylococcus aureus* UFPEDA-709 (presenting the same MIC value when compared to the standard antibiotic) and for *Candida albicans* 4664 and *Candida glabrata* UFPEDA-6393. In addition, none of the evaluated lignins showed minimal bactericidal or fungicidal concentration.

The mechanism of *in vitro* antimicrobial activity promoted by lignins is still not fully elucidated. However, it is known that lignins that present greater amounts of phenolic fragments containing a C-C double bond in the α and β positions of the side chain and a methyl group

in the γ position promote greater inhibition of microbial growth when compared to lignins that present higher levels of phenolic fragments with functional groups containing oxygen (–OH, –CO, COOH) in the side chain (Melo et al. 2020, Araujo et al. 2022).

Araujo et al. (2022) evaluating alkaline lignins obtained from the branches and leaves of *Buchenavia viridiflora* verified that these were able to promote antimicrobial activity, presenting a minimum inhibitory concentration of 512 µg/mL for the bacteria *Enterococcus faecalis* UFPEDA-69, *Staphylococcus aureus* UFPEDA-709, *Pseudomonas aeruginosa* UFPEDA- 261 and *Candida albicans* 4664 yeast. Furthermore, the authors observed that none of the evaluated lignins showed minimal bactericidal or fungicidal concentration.

Melo et al. (2020) evaluating the antifungal activity of a lignin obtained from the leaves of *Caesalpinia pulcherrima* showed different MIC values, among which we can list *Candida parapsilosis* (ATC22019) (31.25µg/mL), *Candida krusei* (ATC62) (125.00 µg/mL) *Candida guilliermondii* (URM62) (31.25 µg/mL), *Candida albicans* (URM49) (250 µg/mL), *Aspergillus flavus* (500 µg/mL), *Aspergillus furmigatus* (500 µg/mL) and two species of *Cryptococcus neoformans* that presented 15.62 µg/mL) respectively. These results show that alkaline lignins can be potential antimicrobial agents.

CONCLUSION

Through the studies carried out, we can conclude that *Morinda citrifolia* lignin is a macromolecule that presents promising results to be applied as an immunomodulatory, antitumor and antimicrobial agent *in vitro*. This dual effect shows that the evaluated lignin can be used as a raw material to obtain products with high added value.

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