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MARCELA DANIELA MUNIZ ARRUDA

Caracterização da lignina das folhas de *Crataeva tapia* e seu potencial em formulações medicinais e cosméticas

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Dissertação apresentada ao Programa de Pós-graduação em Morfotecnologia do Centro de Biociências da Universidade Federal de Pernambuco, para obtenção do título de Mestre em Morfotecnologia.

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Orientador: Profª. Dra. Ivone Antônia de Souza

Co-orientador: Dr. José Iranildo da Cruz

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RESUMO

O Brasil possui a maior diversidade biológica do mundo, possuindo cerca de 55.000,00 espécies registradas, sendo considerado o país mais rico em biodiversidade do mundo. Dentre a vasta biodiversidade brasileira, encontra-se a família Capparaceae, cujas espécies têm amplas propriedades terapêuticas. Nesta pesquisa, destacamos a espécie *Crataeva tapia*, onde obtivemos o processo de extração de lignina a partir das folhas, entretanto estudos relacionados ainda são escassos na literatura científica. As ligninas são potencialmente úteis em uma variedade de aplicações, incluindo o tratamento de diabetes, controle da obesidade, atividade antiviral e proteção solar além de apresentarem possíveis papéis antiinflamatórios e imunomoduladores. O presente estudo avaliou a caracterização da lignina das folhas de *Crataeva tapia* e seu potencial em formulações medicinais e cosméticas. A lignina foi obtida por deslignificação alcalina e sua caracterização físico-química foi feita por meio de FT-IR, UV-Vis, espectroscopia de RMN, análise elementar, determinação de massa molecular e análise térmica. A lignina apresentou baixa atividade antioxidante. A Citotoxicidade foi avaliada por citometria de fluxo e a atividade fotoprotetora foi avaliada pela adição de diferentes concentrações de lignina em um creme comercial. A lignina não foi citotóxica, estimulou a produção de TNF- α, IL-6 e IL-10 e não promoveram alteração significativa nos níveis de óxido nítrico. Portanto, essas descobertas sugerem que a lignina de *C. tapia* tem potencial aplicações farmacêuticas, particularmente cosméticas, aplicadas como aditivo para protetores solares.

Palavras-Chave: Plantas medicinais. Lignina**.** Imunologia**.**

ABSTRACT

Brazil has the greatest biological diversity in the world, having about 55,000.00 registered species, being considered the richest country in biodiversity in the world. Among the vast Brazilian biodiversity, there is the Capparaceae family, whose species have broad therapeutic properties. In this research, we highlight the species Crataeva tapia, where we obtained the process of extracting lignin from the leaves, however related studies are still scarce in the scientific literature. Lignins are potentially useful in a variety of applications, including the treatment of diabetes, obesity control, antiviral activity and sun protection, in addition to having possible anti-inflammatory and immunomodulatory roles. The present study evaluated the characterization of lignin from Crataeva tapia leaves and its potential in medicinal and cosmetic formulations. Lignin was obtained by alkaline delignification and its physical-chemical characterization was done by FT-IR, UV-Vis, NMR spectroscopy, elemental analysis, molecular mass determination and thermal analysis. Lignin had low antioxidant activity. Cytotoxicity was assessed by flow cytometry and photoprotective activity was assessed by adding different concentrations of lignin to a commercial cream. Lignin was not cytotoxic, stimulated the production of TNF-α, IL-6 and IL-10 and did not significantly alter nitric oxide levels. Therefore, these findings suggest that C. tapia lignin has potential pharmaceutical applications, particularly cosmetics, applied as an additive to sunscreens.

Keywords: Medicinal plants. Lignin. Immunology.

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µg	micrograma
µol	micromol
cm	centímetros
FGF-7	Fator de Crescimento de Fibroblastos 7
h	hora
IC50	Concentração Inibitória 50%
IL-1	Interleucina 1
IL-10	Interleucina 10
IL-17	Interleucina 17
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-6	Interleucina 6
L	Litros
mg	miligrama
nm	nanômetros
NMR	Ressonância Nuclear Magnética
NO	Óxido Nítrico
OMS	Organização Mundial de Saúde
PBMC	Monócitos Mononucleares do Sangue Periférico
TNF-α	Fator de Necrose tumoral Alfa
VEGF-A	Fator de Crescimento Endotelial Vascular A

SUMÁRIO

1 INTRODUÇÃO

Segundo a Organização Mundial de Saúde (OMS), as plantas medicinais são definidas como “todo e qualquer vegetal que possui, substâncias que podem ser utilizadas com fins terapêuticos ou que sejam precursores de fármacos semissintéticos” (WORLD HEALTH ORGANIZATION, 1998). Sendo assim, uma fonte variada de produtos naturais biologicamente ativas, muitas das quais são modelos para a síntese de inúmeros medicamentos.

Cerca de 80% da população mundial faz uso da medicina tradicional para o controle e tratamento das mais diversas doenças (FENALTI *et al*., 2016). Embora os estudos nessa área venha ganhando espaço, dados disponíveis revelam que apenas 17% das plantas foram estudadas quanto ao seu potencial medicinal (BARREIRO *et al*., 2019).

Entre a diversidade de família destaca-se a Capparaceae, descrita por Antoine Laurent de Jussieu, apresenta aproximadamente 46 gêneros e 650 espécies espalhadas nas regiões tropicais e subtropicais de todo mundo (SILVA, J.C.S *et al,* 2020), apresenta distribuição pantropical, sendo adaptada em habitats sazonalmente secos, sendo aplicada também aos domínio fitogeográfico da Caatinga, onde a Capparaceae aparece como uma das principais famílias, por possuir um número considerável de espécies de ampla distribuição (NETO, R. L.S *et al*. 2014).

Dentre estas espécies encontra-se *Crataeva tapia*, conhecida popularmente como catoré, cabaceira, cabaceira-do-pantanal e pau-d’alho (SOUSA, 2019). Achados na literatura mostrou que uma proteína isolada a partir da casca dessa espécie, apresentou propriedades biológicas como antiinflamatória, analgésica, antitumoral e com atividade inseticida (ZHANG *et al*, 2013), sendo também utilizada como tônico, febrífugo e no combate das infecções do trato respiratório, devido as propriedades medicinais existentes em suas folhas, cascas e frutos (SHARMA *et al,* 2013), sendo essas propriedades terapêuticas associadas a presença de compostos bioativos.

Os compostos bioativos consistem em polissacarídeos estruturais, como celulose, lignina e pectina que são encontrados na parede celular, os quais são encontrados em

quase todas as paredes celulares vegetais, proporcionando maior ou menor rigidez, dependendo de sua localização no corpo da planta (MOHNEN *et al*., 2008).

As ligninas são potencialmente úteis em uma variedade de aplicações, incluindo o tratamento de diabetes, controle da obesidade, atividade antiviral e proteção solar além de apresentarem possíveis papéis antiinflamatórios e imunomoduladores (MORGANTI, 2017). Esse estudo teve como objetivo avaliar as atividades imunomoduladora e a atividade antioxidadante da Pectina extraída das folhas da *Crataeva tapia*.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Família Capparaceae

Capparaceae abrange cerca de 25 gêneros e aproximadamente 500 espécies (Souza & LORENZI, 2012). Na América Central são citados 18 gêneros e 110 espécies (CORNEJO, 2009) e no Brasil, são reconhecidos 12 gêneros e 28 espécies, das quais 12 são endêmicas, a família apresenta uma ampla distribuição podendo ser encontrada em Áreas Antrópicas, Campo de Várzea, Carrasco, Cerrado, Floresta Ciliar ou Galeria, Floresta de Igapó, Floresta de Terra Firme, Floresta Estacional Decidual, Floresta Estacional Semidecidual, Floresta Ombrófila, Manguezal, Restinga e principalmente na Caatinga no Nordeste Brasileiro (CORNEJO et al., 2015).

A família Capparaceae se distingue por apresentar folhas simples trifoliar, alternadas, estames numerosos, ovários super, fruto capsular e tendo o número de sementes variadas de acordo com a espécie (NEWMAN, 2007).

2.2 *Crataeva tapia* Linn.

Crataeva tapia L. é uma planta nativa que apresenta cheiro semelhante ao do alho em suas folhas, mede cerca de 5-12 m de altura, com copa arredondada e densa, seus frutos são comestíveis, possui características medicinais, ecológicas e econômicas (SANTOS-MOURA et al., 2014), conhecida popularmente como cabaceira, cabeceira, cabaceira-do-pantanal e pau-d'alho, porém é mais conhecida como tapiá ou trapiá, encontrada em Pernambuco principalmente na Zona da Mata e na Caatinga, São Paulo e Minas Gerais, na Mata Atlântica Pluvial e no Pantanal Mato-grossense (TABARELLI; SILVA, 2002).

Na Região do Nordeste brasileiro a *C. tapia* é utilizada como remédio caseiro para diversas patologias (LUCENA et al., 2007), sua casca é utilizada popularmente para o tratamento de diabetes, dor de estômago e febre. A casca da raiz e a casca do caule têm confirmado seus efeitos benéficos nos distúrbios urinários, incluindo urolitíase, e revelou seus potenciais benefícios na diminuição de processo inflamatório, tal como a artrite, além de possuir um importante metabólito secundário, o lupeol, utilizada na medicinal tradicional (SHARMA et al., 2013).

Estudos encontrados na literatura confirmam a presença da lectina CrataBL extraída da entrecascas dessa espécie, que tem propriedade medicinal frente as atividades antitumoral, antiinflamatória e antinociceptiva (ARAÚJO et al., 2011). Segundo estudos realizados por Rocha et al. (2014) confirmam que o CrataBL tem atividade hipoglicêmica, que melhoraram complicações renais e hepáticas do paciente com diabetes.

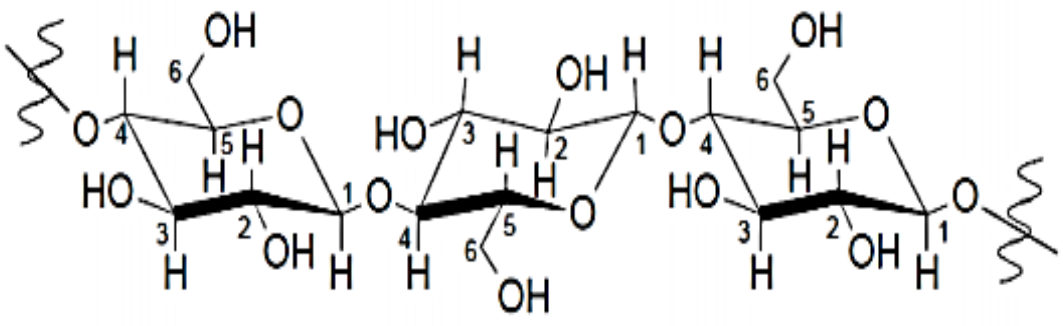
2.3 Composição Química

2.3.1 Celulose

A celulose é um dos componentes mais importantes da parede celular das plantas, é um polímero natural com alta massa molar com unidades de glicose que estão unidas por ligações glicosídicas β (1,4) onde cada unidade de glicose contém três hidroxilas livres ligadas aos carbonos 2, 3 e 6 respectivamente, como ilustrado na figura 1, encontrado em grande quantidade podendo ser obtido de diferentes fontes e com diferentes morfologias. É encontrado em escalas de nanômetros e milímetros, de acordo com o procedimento utilizado para o seu isolamento e sua aplicação (LAVORATTI, 2015).

Suas fontes são diversas, como: biomassa lignocelulósica, exoesqueleto de insetos, madeiras, algumas bactérias, fungos e protoázios, os quais conseguem sintetizar a celulose (RODRIGUES, 2014). Esse polímero pode ser modificado para a obtenção de produtos industrializados, juntamente com seus derivados, a celulose é um material com diversas aplicações podendo ser empregada no campo da medicina, farmácia, indústrias alimentícias, têxtil e na produção de polpa e papel (FIDALE, 2010; LAVAROTTI, 2015).

Figura 1 – Estrutura da Celulose



Fonte: CERQUEIRA, 2009.

2.3.2 Hemicelulose

A hemicelulose é formada por heteropolissacarídeo composto por hexoses (D-glicose, D-galactose e D-manose), pentoses (D-xilose, L-arabinose), ácido acético e ácido Dglucurônico. As pentoses e as hexoses são unidas através de ligações glicosídicas, na maioria das vezes são acetiladas, é uma estrutura hidrofílica que atua como adesivo entre a celulose e a lignina (PATIÑO LAGOS, 2015). A sua classificação é de acordo com os açúcares encontrados na cadeia polimérica principal, podendo ser xilana, glucomanana e galactana (VIEIRA, 2016). Geralmente o heteropolissacarídeo é formado por mais de um tipo desses açúcares e varia de acordo com a planta (SANTOS, 2012).

As diversas ligações, ramificações e unidades monoméricas confirmam a complexidade da estrutura hemicelulósica (FERREIRA, 2015). A hemicelulose se organiza em diferentes estruturas, lineares, nas homoxilanas, e ramificadas, nas heteroxilanas (GRACIOLI, 2018). As homoxilanas são homopolissacarídeos de xilose não ramificados, incluem as β-1,4-D-xilanas, as β-1,3-D-xilanas, e as β-1,3; 1,4-Dxilanas. Estas últimas são pouco comuns em ambientes naturais, podendo ser encontrada em alguns tipos de plantas e algas (GRACIOLI, 2018).

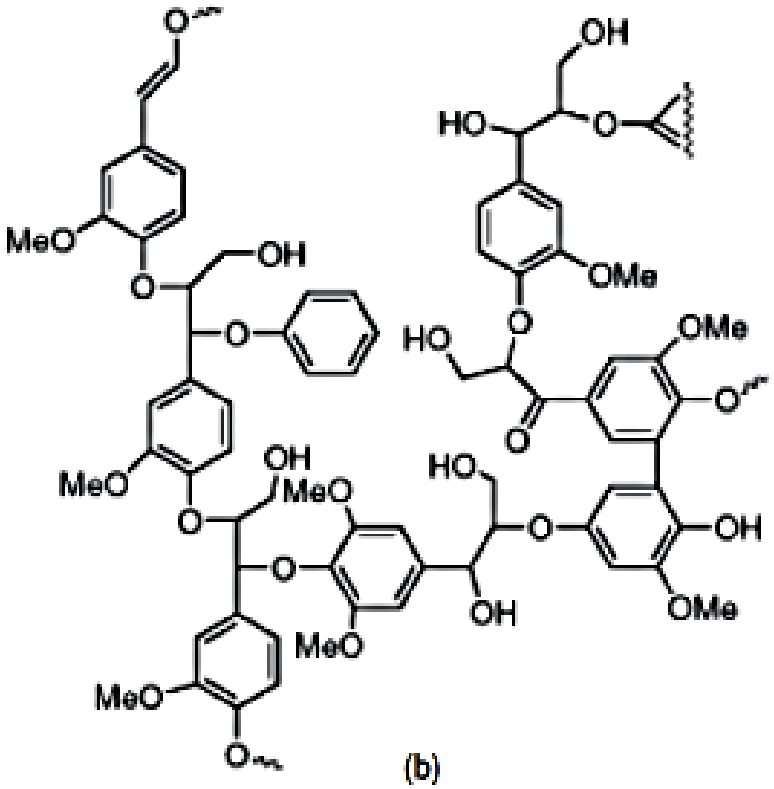
A hemicelulose é diferente da celulose por ser uma estrutura amorfa, tem baixa massa molecular e hidrolisada. Para retirar a fração hemicelulósica dos materiais lignocelulósicos é necessário aplicar alguns tipos de pré-tratamento como hidrólise ácida ou hidrotérmica, para desprender os açúcares das cadeias poliméricas, principalmente a xilose, que posteriormente será fermentada para a fabricação de etanol (VIEIRA, 2016; SILVA, 2010).

2.3.3 Lignina

A lignina é composto por um anel aromático macromolecular gerado através da polimerização de três álcoois fenil-propanos, p-cumarílico, coniferílico e sinapílico como mostra na figura 2 (VIEIRA, 2016). Sua forma é tridimensional, constituída por unidades de propilfenol, com metoxila no anel aromático, ligadas através de ligações do tipo éter estabelecendo ligações cruzadas entre si (SILVA, 2010).

Estas estruturas não tem relação com moléculas de açúcar, portanto não é utilizada na produção de bioetanol por rotas fermentativas. Mesmo em pequena quantidade a lignina retarda ou impede completamente o processo de sacarificação, importante para a produção de etanol, por isso é necessário um pré-tratamento do material com lignina (FERREIRA, 2015).

Figura 2- Estrutura da lignina



Fonte: Adaptado de SILVA (2010) e FERREIRA (2015).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a caracterização da lignina das folhas de *Crataeva tapia* e seu potencial em formulações medicinais e cosméticas

3.2 OBJETIVOS ESPECÍFICOS

- Coletar e Identificar o material botânico;
- Preparar o Extrato das folhas da *Crataeva tapia*;
- Avaliar o Perfil Fitoquímico das Folhas da *Crataeva tapia*;
- Extrair a Lignina das Folhas da *Crataeva tapia*;
- Avaliar a Atividade Antioxidante da Lignina extraída das Folhas da *Crataeva tapia*;
- Avaliar o Potencial Imunomodulador da Lignina extraída das Folhas da *Crataeva tapia*;
- Realizar os Ensaios de Imunofenotipagem da Lignina extraída das folhas da *Crataeva tapia*;
- Avaliar a Atividade de Fotoproteção *in vitro* das Folhas da *Crataeva tapia*.

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5 ARTIGO

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Characterization of a lignin from *Crataeva tapia* leaves and potential applications in medicinal and cosmetic formulations

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ABSTRACT

Lignins are phenolic macromolecules that have several applications. In this work, we examine some biological activities of a lignin-like macromolecule isolated from the *Crataeva tapia* leaves, not yet studied to evaluate its potential applications in medicinal and cosmetic formulations. Lignin was obtained by alkaline delignification and its physical-chemical characterization was made by means of FT-IR, UV–Vis, NMR spectroscopy, elementary analysis, molecular mass determination and thermal analysis. Lignin is of the GSH type, with levels of hydrogen (5.10%), oxygen (27.18%), carbon (67.60%), nitrogen (0.12%) and phenolic content of 189.6 ± 9.6 mg GAE/g. In addition, it is a thermally stable macromolecule with low antioxidant activity. Cytotoxicity and cytokine production were assessed by flow cytometry. The photoprotective activity was evaluated by adding different concentrations of lignin to a commercial cream. Lignin was not cytotoxic, it stimulated the production of TNF-α, IL-6 and IL-10 and did not promote a significant change in nitric oxide levels. In addition, this macromolecule was able to promote increased absorption of ultraviolet light from a commercial cream. These results reinforce the ethnopharmacological use of *C. tapia* leaves and suggest the need for further studies to determine the potential medicinal and cosmetic applications (sunscreen) of lignin from *C. tapia* leaves.

1. Introduction

Lignins are one of the main macromolecules that confer resistance and shape to the plant cell wall [1]. These macromolecules are irregular, polyphenolic polymers synthesized by dehydrogenation polymerization of phenylpropanoid units, *para*-coumaryl (*p*-coumaryl) alcohol, coniferyl alcohol, and sinapyl alcohol, to yield *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) structures, respectively [2]. We can also observe the existence of lignin-like macromolecules because they contain the same functional groups and chemical bonds [3].

Lignins are potentially useful in a variety of applications, including the treatment of diabetes [4], obesity control [5], antiviral activity [6], anti-phenysphysema activity [7] and sun protection [8], in addition to having possible anti-inflammatory and immunomodulatory roles [9]. Lignin-carbohydrate complexes (LCCs) have shown important biological activities, these can act as antioxidants to eliminate reactive oxygen species *in vitro* and *in vivo* [10], as immunostimulants [11], in addition to promoting anti-inflammatory, anti-tumorigenic activities and anti-protein aggregation [12]. In addition to these biological activities, lignins are excellent thermoplastics and can be used as an excipient for the controlled release of drugs [13,14]. This wide range of activities has generated interest in biorefining these macromolecules for biotechnological uses [1–7].

The extraction of phytochemicals from plants generates a large amount of residual biomass that is usually burned in low-energy boilers to produce process steam or is added to landfills [7,15]. The rational use of this residue to produce lignin and cellulose is therefore of great economic and environmental interest [7]. The isolation of bioactive molecules from the leaves is an attractive alternative, due to the large amount of leaves produced when compared to the stem. In addition, these can be easily recovered during pruning [16].

Crataeva tapia (family Capparaceae) is a plant species native to the Americas, with a wide distribution from Mexico to the Amazon basin [17,18]. Various studies have shown that extracts from different *C. tapia* tissues have antioxidant, antinociceptive, wound healing, antidiarrheal and anti-inflammatory activities, in agreement with the widespread use of these plants in herbal medicines [19].

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C. tapia lignin has not yet been studied, therefore, in this work, we report obtaining lignin and examining its physical-chemical properties. We also studied its antioxidant, cytotoxic, immunostimulant and photoprotective activities as part of an assessment of its potential usefulness in medicinal and cosmetic formulations, since lignins in general have not yet been converted into high-value products on a large scale.

2. Material and methods

2.1. Reagents

Acetic anhydride (CAS 108247), ascorbic acid (CAS 50-81-7), dimethylsulfoxide (DMSO; CAS 67-68-5), 2,2-diphenyl-1-picrylhydrazyl (DPPH; CAS 1898-66-4), Griess reagent, KBr (CAS 7758-02-3), pyridine (CAS 110861), Sephadex G-50 and trichloroacetic acid (CAS 76-03-9) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; CAS 30931-67-0), ethanol (CAS 64-17-5), $FeCl_3$ (CAS 10025-77-1), Folin-Ciocalteu reagent, H_2SO_4 (CAS 7664-93-9), Na_2CO_3 (CAS 497-19-8), NaOH (CAS 1310-73-2), potassium persulphate (CAS: 7727-21-1), toluene (CAS 108-88-3), potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_20$] (CAS14459-95-1), Albumin (CAS:9048-46-8), Carbonic anhydrase (CAS 9001-03-0), Cytochrome C (CAS 9007-43-6) and Aprotinin (CAS 9087-70-1) were obtained from Merck Millipore (Rio de Janeiro, RJ, Brazil). H_3PO_4 (CAS 7664-38-2) was from Labsynth (São Paulo, SP, Brazil). Nitrogen was supplied by White Martins (Pernambuco, PE, Brazil).

2.2. Plant materials

Adult phase *Crataeva tapia* leaves were collected in January 2020, in the city of Recife, Pernambuco state, northeastern Brazil (8° 2′50,312″S, 34° 56′ 58,364″ W). Healthy green leaves that were visually intact and free of pests, diseases or discoloration were used. The material was dried in a three-phase forced circulation oven for 48 h at 65 °C and then ground in a knife mill (Fritsch-pulverisette 14) and sieved with a 100-mesh granulation sieve.

2.3. Analysis of the composition of the leaves of Crataeva tapia

The leaves of *Crataeva tapia* were analyzed for physical and chemical compositions (cellulose, hemicellulose, lignin, extracts and ashes) according to the analytical methodology proposed by Rocha et al. [20].

2.4. Extraction of lignin from C. tapia leaves

Lignin was obtained using an adaptation of the method described by Cruz-Filho et al. [21]. Extraction of the leaves was done in three stages. In step I, a 300 g sample of leaves was extracted in a Soxhlet extractor using a toluene/ethanol mixture (32:68, v/v) for 6 h to remove compounds that could interfere with the chemical structure of the lignin. In step II, the solid fraction from the preceding step was subjected to acid hydrolysis in 1% H_3PO_4 (solid:liquid ratio, 1:10, w/v) in an autoclave at 121 °C for 1 h to remove carbohydrates from the hemicelluloses. In step III, the solid material containing essentially cellulose and lignin underwent alkaline delignification with 1% NaOH under the same conditions as acid hydrolysis. At the end of the process the lignin-rich black liquor was separated from the cellulose by filtration. After filtration, the black liquor was acidified with H_2SO_4 to pH 2 to precipitate the lignin and the mixture was incubated at 30 °C for 12 h without stirring. After this period, the lignin was filtered and carefully washed until neutralized and then dried at 70 °C for 24 h. The yield of obtaining lignin was obtained by

$$Y\,(\%) = \left(\frac{\text{Mass of lignin extracted}}{\text{Total lignin mass in leaves}}\right) * 100\%. \quad (1)$$

2.5. Physical and chemical characterization of lignin

2.5.1. Fourier-transform infrared (FT-IR) analysis

To identify the main functional groups, lignin was subjected to FT-IR analysis in a Bruker IFS66 spectrophotometer to obtain the vibrational spectrum in the infrared region (4000 to 400 cm^{-1}). The samples were prepared in KBr.

2.5.2. Spectroscopic analysis in the ultraviolet-visible (UV–Vis) region

UV–Vis spectroscopy was done as described by Rocha et al. [20], with slight modifications, using a Hewlett-Packard spectrophotometer (model 8453) over a wavelength range of 250–280 nm. The sample (0.03 g of lignin/L) was solubilized in 0.01 mM NaOH, pH 12; the blank consisted of NaOH solution without lignin. The extinction coefficient was determined over a concentration range of 0.1–0.02 g/L in 0.01 mM NaOH.

2.5.3. Acetylation of lignin and magnetic resonance analysis (^1H and ^{13}C)

For NMR analyses (^1H and ^{13}C), the sample was initially acetylated to increase its solubility. Lignin (30 mg) was dissolved in 5 mL of pyridine and 5 mL of acetic anhydride followed by bubbling with nitrogen for 15 min and then a 50 h incubation in the dark at room temperature. At the end of this incubation, the remaining acetic anhydride was removed by adding 4 mL of methanol. The solvent was evaporated under reduced pressure and was facilitated by the addition of toluene and ethanol to form a mixture of azeotropes. The acetylated lignin was subsequently freeze-dried and stored in a vacuum desiccator. Once lignin is acetylated, it becomes totally soluble in organic solvents [20,22]. For ^1H and ^{13}C nuclear magnetic resonance (NMR), 20 mg of acetylated lignin was dissolved in $CDC1_3$ followed by analysis in a Varian Plus spectrophotometer (VNMRS400) at 400 MHz and 100 MHz.

2.5.4. Elemental analysis

The levels of carbon, hydrogen, nitrogen and oxygen present in the lignin structure were determined with an element analyzer (Perkin Elmer 2400 Series II).

2.5.5. Analysis of lignin molar mass distribution by gel permeation chromatography (GPC)

Gel permeation chromatography was done as described by Rocha et al. [20] and Melo et al. [23]. The chromatographic system consisted of a glass column (57 cm × 1.8 cm) packed with Sephadex G-50 equilibrated in 0.5 M NaOH. The column was loaded with sample (0.4 mL of a solution containing 2 mg of Lignin/mL) and eluted with 0.5 M NaOH at a flow rate of 0.4 mL/min. Fractions of 4 mL were collected and the absorbance was determined at 280 nm with a Hewlett- Packard® spectrophotometer against a blank of NaOH solution. The column was calibrated with marker proteins of known molecular mass (albumin - 66 kDa, carbonic anhydrase - 29 kDa, cytochrome C -12.4 kDa, aprotinin - 6.5 kDa) and sugarcane bagasse lignin (2.0 kDa, 95% purity) and was used to determine the number average molecular weight (Mn), weight average molecular weight (Mw) and Mw/Mn ratio (dispersibility).

2.5.6. Thermal analysis of lignin

Thermogravimetric analysis (TGA) was done using a Perkin Elmer model STA 6000 apparatus. The analyses were done under a nitrogen gas atmosphere at a constant flow rate of 20 mL/min, using ~20 mg of sample in an open alumina capsule. The thermogravimetric curves were obtained over the temperature range of 25–600 °C, at a heating rate of 10 °C/min. A thermogravimetric curve was used to evaluate the loss of mass as a function of temperature and the curve of the first loss of mass derivative (derivative thermogravimetry – DTG). Differential scanning calorimetry (DSC) was done under the same conditions as TGA.

2.5.7. Determination of total phenols

The total phenol content was determined by the Folin-Ciocalteu method according to Santos et al. [24], with some modifications. The test was done using 0.2 mL of lignin solution (1 mg/mL) dissolved in 10% DMSO added to 2 mL of Folin-Ciocalteu solution (1:10 v/v). After a 4 min incubation in the dark, 1.6 mL of 7.55% Na_2CO_3 was added and incubated for 2 h in the dark at room temperature, after which the absorbance was read at 765 nm. The blank consisted of reagent and distilled water. A standard curve was prepared by plotting the absorbance as a function of the concentration of gallic acid (0–100 μg/mL) and then determining the linear equation ($y = 0.0023\times + 0.014$; $R^2 = 0.9858$). The phenol content was expressed in gallic acid equivalents (mg GAE/g of lignin).

2.6. In vitro antioxidant activity

2.6.1. Total antioxidant activity test (TAA): reduction of the phosphomolybdenum complex

The ability of lignin to reduce the phosphomolybdenum complex was determined according to Cruz-Filho et al. [21]. Lignin was dissolved in 10% DMSO and diluted to concentrations ranging from 3.9–500 μg/mL. To perform the test, a volume of 0.3 mL of each lignin solution (in concentrations 3.9–500 μg/mL) was added to 3 mL of phosphomolybdenum solution then the reaction system was maintained at 95 °C for 90 min and the resulting absorbance was determined at 695 nm. The blank consisted of 3 mL of phosphomolybdenum solution and 0.3 mL of distilled water. The antioxidant standard used in this experiment was ascorbic acid. The antioxidant activity was calculated using the following

$$\text{TAA}\ (\%) = [(\text{As}-\text{Ac}) \div (\text{Aaa}-\text{Ac})] \times 100 \quad (2)$$

where Ac = control absorbance, As = sample absorbance and Aaa = ascorbic acid absorbance.

2.6.2. DPPH assay

The assay is based on the transfer of electrons by an antioxidant substance in which 2,2-diphenyl-1-picryl-hydrazine (DPPH, purple in color) is reduced to diphenyl-picryl-hydrazine (yellow in color) [21,24]. The lignin was solubilized in 10% DMSO and diluted to 3.9–500 μg/mL. The assay was done by adding 2.5 mL of 1 mM DPPH to 0.2 mL of lignin solution followed by incubation for 25 min in the dark, after which the absorbance at 517 nm was read. The control consisted of DPPH added to 0.2 mL of water and the assay blank was ethanol. Ascorbic acid was used as the antioxidant standard. The sequestration of DPPH radicals was calculated using the

$$[\text{DPPH}]\ (\%) = [(\text{As}-\text{Ac}) \div \text{Ac}] \times 100 \quad (3)$$

where As = sample absorbance and Ac = control absorbance.

2.6.3. ABTS assay

Free radical consumption by the lignin was also assessed based on the discoloration of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as described by Barapatre et al. [25]. A stock solution of 7 mM ABTS was prepared in 2.45 mM potassium persulphate. For the assay, lignin (100 μL of dilutions ranging from 3.9–500 μg/mL prepared in 10% DMSO) was added to 3 mL of ABTS and the resulting absorbance was read at 734 nm. The assay control consisted of ABTS without lignin and ascorbic acid was used as an antioxidant standard; the assay blank was methanol. The sequestration of ABTS radicals was calculated using the

$$[\text{ABTS}]\ (\%) = [(\text{As}-\text{Ac}) \div \text{Ac}] \times 100 \quad (4)$$

where As = sample absorbance and Ac = control absorbance.

2.6.4. Nitric oxide radical scavenging assay

Nitric oxide (NO) sequestration by the lignin was assayed as described by Barapatre et al. [25], with modifications. The lignin was solubilized in 10% DMSO and diluted to 3.9–500 μg/mL. The assay was done by adding 1 mL of Griess reagent to 0.2 mL of lignin followed by incubation for 15 min at room temperature (25 °C), after which the absorbance was read at 540 nm. The sequestration of NO radicals was calculated using the

$$[\text{NO}]\ (\%) = [(\text{As}-\text{Ac}) \div \text{Ac}] \times 100 \quad (5)$$

where As = sample absorbance and Ac = control absorbance.

2.6.5. Iron reduction

Lignin (3.9–500 μg/mL, diluted in 10% DMSO) was added 0.5 mL to 0.5 mL of 0.2 M phosphate buffer, pH 6.6, containing 1% potassium ferrocyanide [$K_2Fe(CN)_6$] followed by incubation at 50 °C for 20 min. After this incubation, 0.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged (3000 rpm, 10 min). An aliquot (0.5 mL) of the resulting supernatant was mixed with 2 mL of distilled water plus 50 μL of 0.1% $FeCl_3$ and the absorbance was read at 700 nm. The reduction was calculated using the

$$[\text{Iron reduction}]\ (\%) = [(\text{As}-\text{Ac}) \div \text{Ac}] \times 100 \quad (6)$$

where As = sample absorbance and Ac = control absorbance.

2.7. Cytotoxicity and immunomodulation of human peripheral blood mononuclear cells (PBMC)

2.7.1. Preparation, culture and stimulation of human PBMC

Lymphocytes and monocytes were prepared from human peripheral blood mononuclear cells (PBMC) as described by Santos et al. [24]. The use of these cells from human donors was approved by the Ethics Committee of the Federal University of Pernambuco (protocol no. 1.870.360/2016). The cells were isolated using a Ficoll® 1.077 gradient (Sigma-Aldrich) and were counted in a Neubauer chamber using trypan blue solution. Cells were only used when viability was >95%. PBMC were cultured in RPMI 1640 medium supplemented with 10% (w/v) fetal bovine serum containing 1% of antibiotics penicillin and streptomycin in 24-well plates at a density of 10^6 cells/well. For the viability and immunostimulant assays, the cells were incubated with different lignin concentrations for 24 h.

2.7.2. Cytotoxicity

The PBMC were incubated with lignin (2.5–80 μg/mL) for 24 h and cell death was assessed using propidium iodide in a FACS Calibur® flow cytometer (BD Biosciences). This test is based on the ability of the propidium iodide dye to bind to DNA emitting high fluorescence when excited by the laser. Cells with an integral membrane do not allow the dye to enter, so they have low fluorescence. However, when the membrane is broken, it allows the dye to enter, emitting high fluorescence. The cellular viability was calculated using the

$$[\text{Cellular viability}]\ (\%) = [(\text{VC} \div \text{TC}] \times 100 \quad (7)$$

where VC = viable cells and TC = total cells. The results were analyzed using Flowing Software 2.5.1®. The experiment was performed six times.

2.7.3. Cytokine and nitric oxide production

Cytokine and nitric oxide (quantified as nitrite) production were assayed in supernatants of PBMC incubated with lignin for 24 h. For this test, a single concentration (10 μg/mL) of lignin was used. Santos et al. [24] evaluating the immunological activity of lignin isolated from the leaves of *Conocarpus erectus*, found that this concentration was sufficient to induce an immune response in PBMC cells.

Cytokines were quantified using the Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine kit (BD Biosciences) designed to detect interleukins IL-2, IL-4, IL-6, IL-10 and IL-17a, TNF-α and IFN-γ, as described by Santos et al. [24]. The data were acquired using a FACS Calibur® platform (BD Biosciences) and the results were analyzed using Flowing Software 2.5.1®. Nitrite concentrations were estimated by the Griess method [26] in conjunction with a standard curve (3.12–400 μmoL; $y = 0.007x + 0.0256$; $R^2 = 0.9978$). The absorbances were read in a microplate spectrophotometer (Thermo Scientific Multiskan FC®, Waltham, USA) at 595 nm.

2.8. In vitro photoprotection assay

The photoprotection assay was based on a spectrophotometric method, as done in other similar studies with lignins [27,28]. The lignin (1, 2, 5 and 10%) was dissolved in 10% DMSO and incorporated into a neutral non-ionic cream base used in dermocosmetic formulations by compounding pharmacies (DERMABASE cream CNA) at a ratio of 1:10 (v/v). The formulations were diluted in ethanol to a concentration of 0.2 μL/mL.

The absorbances were determined in a Hewlett-Packard® spectrophotometer using a quartz cuvette with a 1 cm optical path, over the range of 290–320 nm at 5 nm intervals. Ethanol was used as the blank. The experiments were done in triplicate and the results were expressed as the mean ± SD. The protection factor was determined por Mansur et al. [29], Sayre et al. [30] and Rincón-Fontán et al. [31] using the

$$FPS_{invitro} = CF \times \sum E\lambda \times S\lambda \times Abs\lambda \quad (8)$$

where $CF\,(=10)$ is an empirical correction factor of the *in vitro* method, $E\lambda$ is the parameter of erythematous spectral effectiveness, $S\lambda$ is the solar spectral irradiance and $Abs\lambda$ is the absorption of UV radiation for a given wavelength. The products $E\lambda.S\lambda$ were previously calculated by Sayre et al. [30], as shown in Table 1.

2.9. Statistical analysis

The numerical results were expressed as the mean ± SD of at least three independent determinations. Statistical comparisons were done using one-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. A value of $\alpha < 0.05$ (5% probability, or $p < 0.05$) indicated significance. All data analyses were done using GraphPad Prim 5.01 software.

3. Results and discussion

3.1. Analysis of the composition of leaves and lignin of Crataeva tapia

The leaves are made up of different levels of cellulose, hemicellulose, lignins, extracts and ash [32]. The components present in the leaves of *Crataeva tapia* in greater quantity were cellulose (35.0 ± 1.2%), hemicellulose (23.4 ± 0.9%) and extracts 19.1 ± 1.3%. In smaller quantities, lignin was 17.3 ± 0.1% and ashes 4.7 ± 0.5%. In the first stages of fractionation, polar and nonpolar bioactive compounds (Stage I and II) were removed, resulting in a lignocellulosic solid. Then the lignocellulosic solid was subjected to acid hydrolysis to remove the hemicelluloses (Step III). At the end of the hydrolysis, a solid containing only cellulose and lignin (cellulignin complex) was obtained. Finally (Step IV), the cellulignin complex was subjected to alkaline delignification, which allowed the lignin to be obtained. The extraction yield of lignin was 85.7%.

Elementary analysis is a technique capable of verifying the amount of carbon, hydrogen and nitrogen. The oxygen content by the mass difference of the mass percentages of the atoms cited by the mass of the sample [22]. Table 2 shows the elemental composition (carbon, hydrogen, nitrogen and oxygen) of lignin from *C. tapia* when compared with others lignins. These results confirm that the extraction method and the source of obtaining directly interfere in the chemical structure of lignins [33].

3.2. Physicochemical characterization of lignin

3.2.1. Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy analysis aims to qualitatively observe the functional and aromatic groups of lignin. Fig. 1 shows the FT-IR spectrum of lignin extracted by delignification. The band assignments were based on those previously determined using other lignins [34–37]. The band between 3700 and 3000 cm^{-1} was attributed to the O—H stretch. The bands close to 2930 and 2810 cm^{-1} were attributed to the C—H stretches of groups CH_3 and CH_2, and the absorption band at 1705 cm^{-1} was assigned to the carbonyl groups. The bands between 1600 and 1500 cm^{-1} were attributed to vibrations of the aromatic rings present in lignin. The bands between 1460 and 1030 cm^{-1} were attributed to C—H deformations of the aromatic ring. The band at 1471 cm^{-1} was attributed to aromatic ring vibrations and C—H deformation of the methoxy groups. The band at 1328 cm^{-1} corresponds to the condensed guaiacyl (G) and syringyl (S) rings. The band at 1168 cm^{-1} is characteristic of GSH-type lignins, and the region of 1140–1100 cm^{-1} corresponds to the C—O deformation of a primary alcohol. The band at 835 cm^{-1} was assigned to the out-of-plane C—H strain at positions 2 and 6 of the guaiacyl and syringyl units, and at all positions of the *p*-hydroxyphenyl unit.

Lignin was obtained by the acid hydrolysis process followed by soda pulping, which is quite different from commercial kraft lignin. The FT-IR spectra of kraft lignins generally show a weak broad band at about 630 cm^{-1} that is attributable to the C—S group and a sharp band at 655 cm^{-1} that is attributable to SO_3H, both of these being characteristic of lignosulfonates [38,39]. Overall, soda lignins (extracted only with NaOH) are purer and more easily depolymerized and more chemically reactive [40,41].

3.2.2. UV–Vis spectroscopy

UV–vis spectra allow identification of the main groups present in the lignin structure and also provide an important tool for determining the

Table 1
Relationship between wavelength (290–320 nm) and $E\lambda.S\lambda$ values.
Data from Sayre et al. [30].

Wavelength (nm)	$E\lambda.S\lambda$
290	0.015
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.018
Total	1

Table 2
Elementary analysis of *C. tapia* lignin and different lignins found in the literature.

Plant	Lignin	C	O	H	N	Reference
Crataeva tapia	**Alkaline**	**67.60**	**27.18**	**5.10**	**0.12**	**This study**
Corn stalk	Klason	60.87	33.15	4.62	1.36	[33]
Corn stalk	Alkaline	60.70	32.53	5.34	1.43	[33]
Corn stalk	Organosolv	67.75	23.23	6.31	2.71	[33]
Poplar	Organosolv	66.87	25.64	6.30	1.20	[33]
Basswood	Organosolv	67.60	25.68	5.93	0.79	[33]
Fir	Organosolv	68.06	26.49	5.13	0.32	[33]
Pine	Organosolv	66.78	28.37	4.85	0.00	[33]

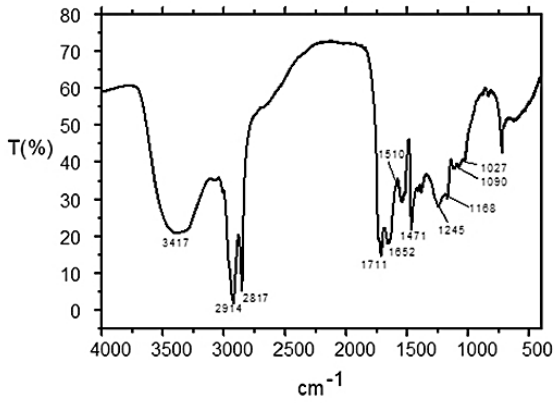

Fig. 1. FT-IR spectrum of *C. tapia* lignin.

purity and analysis of guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units [22,42,43]. The UV/Vis spectrum for lignin (Fig. 2A) indicated absorption between 250 and 600 nm with an absorption peak around 280 nm.

This result for lignin agrees with findings reported for other lignins [20,21,24,36,41]. The band between 250 nm and 280 nm is related to the chromophore groups of conjugated and non-conjugated phenolic compounds, in addition the peak absorbance at 280 nm. Natural lignins have different proportions of G, S and H and this variation in composition is related to the extraction method and species from which the lignin is obtained. The 320 to 450 nm region present in the spectrum is related with ferulic acid and *p*-cumaryl chromophore groups. All bands described have electronic transition π- π * [16,44]. The extinction coefficient is an important parameter that can be used for the quantitative determination of lignin in solution and to infer the extent of lignin condensation. Fig. 2B shows the relationship between lignin concentration and absorbance used to determine the extinction coefficient. Table 3 shows the extinction coefficient for lignin and compares it with other lignins, Melo et al. [23], Rocha et al. [20] Varanasi et al. [45], Oliveira et al. [46] and Rocha et al. [47]. The differences in the extinction coefficients reflect the degree of condensation and substitution in these lignins. According to Rocha et al. [20], the greater the number of replaced rings the greater the absorbance at 280 nm.

3.2.3. Nuclear magnetic resonance (NMR)

Acetylated lignin was solubilized in $CDCL_3$ and submitted to NMR analysis, using ^1H and ^{13}C nuclei. Fig. 3 shows the ^{13}C NMR spectrum, the signals of which have previously been attributed by Cruz-Filho et al. [21], Santos et al. [24], Shi et al. [48] and Kringstad and Mörck [49]. The peak at 167 ppm was attributed to the presence of aromatic groups [21,48], while that at 153 ppm reflects bonds in the syringyl (S) unit [49]. The peak at 145.7 ppm refers to the guaiacyl unit (G) [24], while the peak at 131 ppm represents connections in *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units [24]. The signals at 126 ppm and 116 ppm are associated with ester bonds in *p*-cumaryl acid [21]. The signal at 73 ppm refers to C-α in β-O-4 bonds [43]. The peak at 56 ppm refers to bonds involving -OCH_3 [21,48]. Table 4 shows the assignment of the signals present in *Crataeva tapia* lignin.

Fig. 4 shows the spectrum of ^1H NMR, whose signals were also previously determined for different lignins [20,22,24,41]. The relative percentage of signals present in the spectrum is shown in Table 5.

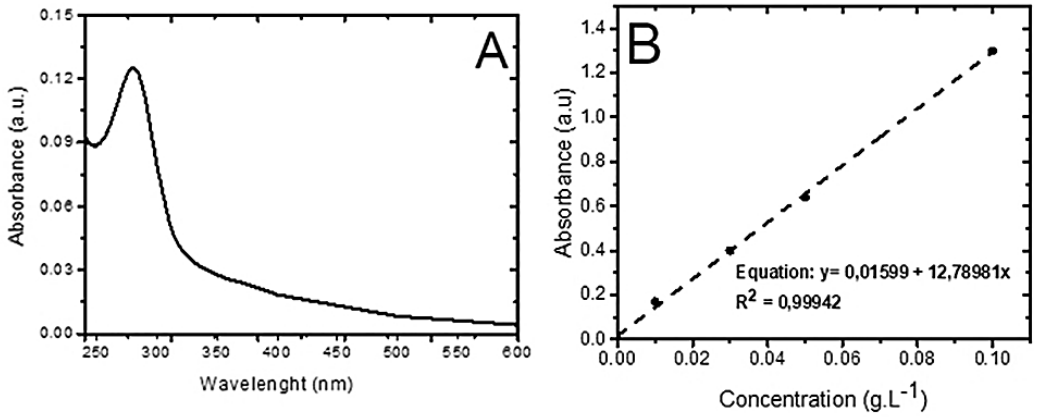

Fig. 2. UV/Vis spectrum (A) and coefficient of extinction (B) of *C. tapia* lignin.

Table 3
Extinction coefficients for different lignins.

Biomass	Process used to obtain the lignin	Absorptivity (L/g/cm)	Reference
Crataeva tapia	**Acid hydrolysis followed by alkaline delignification**	**12.8**	**This study**
C. pulcherrima	Acid hydrolysis followed by alkaline delignification	22.27	[23]
Sugarcane bagasse	Enzymatic depolymerized alkaline lignin	14.6	[20]
Eucalyptus globulus	Lignin obtained by ionic liquid	20.09	[45]
Banana	Acidolysis	18	[46]
Mimosa hostilis Benth	Lignin kraft	21.48	[47]
Sugarcane bagasse	Organosolv oxygen lignin	24.91	[47]
Sugarcane bagasse	Soda-anthraquinone	25.50	[47]
Sugarcane bagasse	Ammoniated lignin	32.22	[47]

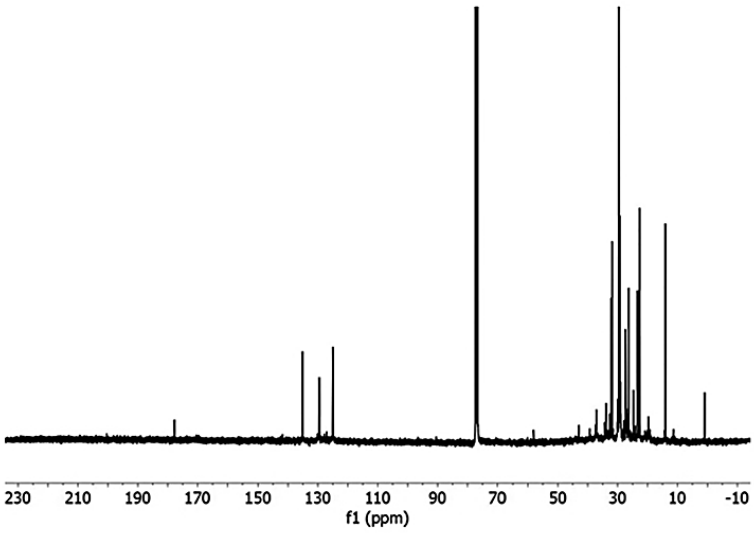

Fig. 3. ^{13}C NMR spectrum of acetylated *C. tapia* lignin.

The chemical shift from 8.0 ppm to 11.5 ppm represents the bonds of carboxylics and aldehydes. Signals in the 6.28–8.0 ppm range are attributed to aromatic protons. Signs observed at 6.6 and 6.9 ppm are attributed to aromatic protons in structures *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S). Signs in the region at 7.7–7.5 ppm confirm the presence of *p*-hydroxyphenyl and Cα═O groups. The aromatic proton content of *C. tapia* lignin was 3.1% (relative percentage of region 6.28–8.0 ppm, Table 5). Garcia et al. [50] studying lignins obtained from the apple residue through different pretreatments and fractionation methods obtained aromatic proton values ranging from 3.0 to 12.69%. Santos et al. [24] and Melo et al. [23] characterizing lignins isolated by alkaline delignification of the leaves of *C. erectus* and *C. pulcherrima* obtained aromatic contents around 7.3 and 3.0%. The signals at 5.74–6.28 ppm refer to the presence of β-vinyls and benzis (Hα of β-O-4 and β-1 structures) and signals of 5.18–5.74 ppm are attributed to Hα, Hβ in substructures β-5. In 4.0–5.1 signals attributed to the protons in the connections of the inter-lignin units β-O-4 [49].

Table 4
Signal assignment in the ^{13}C NMR spectrum of *C. tapia* lignin.

Range δ (ppm)	Assignment
173	C = O
166.5	-COO- in ferulic acid and p-coumaric acid esters
152.1	C-3/C-5 in syringyl etherified, C-3/C′-3 in 5-5′ etherified
145.7	C-α in ferulic acid and p-coumaric acid esters, C-4 in guaiacyl not etherified
131	C-2/C-6 in ferulic acid and p-coumaric acid ethers
125.2	C-α in coniferyl alcohol
115.8	C-3/C-5 in ferulic acid and p-coumaric acid esters, C-3/C-5 in H, C-5 in guaiacyl
83.1	C-β in β-O-4, C-α in β-5 and β-β
72.0	C-α in β-O-4
66.0	C-γ in β-5
61.8	C-5 in xyl internal unit
60.4	C-γ in β-O-4
55.9	-OCH_3
28.1	-CH_2-(C_5-CH_2-C_5)
24.3	-CH_3 in acetyl group
13.2	γ-Methyl in n-propyl side chain

The region of 3.5–4.0 refers to H in methoxyls, a sign presented by ^{13}C NMR (76 ppm). The methoxyl content was 12.8% (Table 5), this result is consistent with those obtained for different lignins. Thakur and Thakur [51], presented results that varied from 10 to 19% for the lignins Soda (bagasse), Soda (wheat straw), Kraft (softwood), Organosolv (hardwood) and Organosolv (bagasse). Santos et al. [24] isolating lignins from the leaves of *C. erectus* obtained a methoxyl group content of 9.6%. The signals at 2.10–2.50 ppm are attributed to aromatic acetoxylics and those at 1.58–2.10 to diphatic acetoxylics and aromatic acetoxylics, while the 0.38–1.53 ppm range of aliphatic groups (C-CH_2-C, -CH_3). The structural differences between the lignins are associated with the source of the lignin and the method of extraction.

The results presented in Table 5 show that *C. tapia* lignina has higher levels of aliphatic groups evidenced by the regions 4.5–5.2 and 0.0–1.6 ppm. This fact may be related to the condensation of lignin fragments during acid precipitation [3]. These results were confirmed by Ghatak et al. [52] who found that lignins obtained by acid precipitation of black liquor had higher levels of hydrogen bonded to β carbon, as well as hydrogen in the non-oxygenated aliphatic region. This fact justifies the low content of aromatics, since the macromolecule obtained has a greater extension of substituent groups and bonds through the aromatic ring [52,53]. Thus, structural differences found for lignins are directly related to the source of production and mainly to the extraction method. [50,51].

3.2.4. Gel permeation chromatography: molar distribution

C. tapia lignin presented weight average molecular weight (Mw) of 1246.8 Da, numerical average molecular weight (Mn) of 831.2 Da and polydispersity value of 1.5. Other authors have reported values similar to these for other lignins. Zhang et al. [54], who studied eucalyptus lignins isolated by the methylisobutyl ketone-water-$FeCl_3$, reported values of Mw, Mn and polydispersity of 1227 Da, 767 Da and 1.76, respectively. Yoya and Stevanovic [55] reported values of Mw, Mn and polydispersity of 1642 Da, 956 Da and 1.7, respectively, for a *Populus tremuloides* lignin obtained by the ethanol-water-$FeCl_3$ method. Similarly, Park et al. [56] reported values of Mw, Mn and polydispersity of 2813 Da, 970 Da and 2.9, respectively, for kraft lignin (commercial); these values were higher than those obtained here for the *C. tapia* lignin. Thakur and Thakur [51], presented results that varied from 800 to 3000 Da for lignins Soda

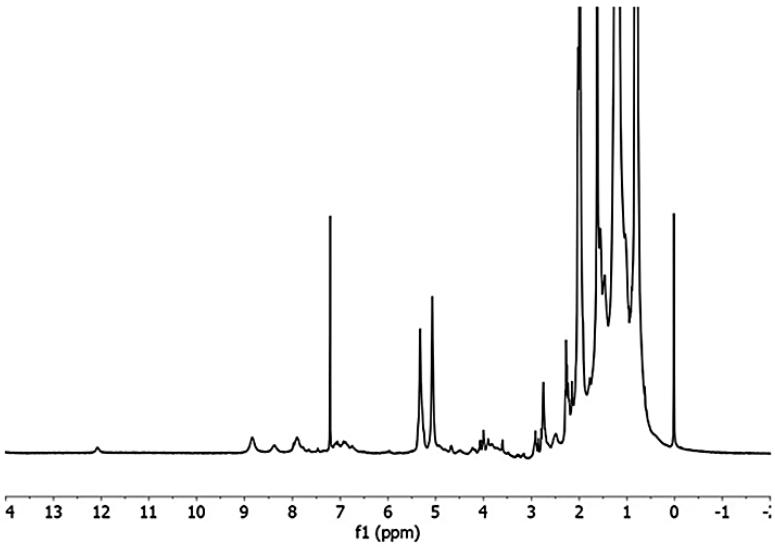

Fig. 4. ^{1}H NMR spectrum of acetylated *C. tapia* lignin.

(bagasse), Soda (wheat straw), Kraft (softwood), Organosolv (hardwood) and Organosolv (bagasse).

The molecular weight of lignins is related to the degree of condensation, that is, the amount of C—C bonds that form the aromatic skeleton [54,57,58]. *C. Tapia* lignin presented low molecular weight, this result corroborates with the results obtained by the UV/Vis analysis, which revealed that lignin has a low degree of condensation. This fact was also observed by the results of NMR analyzes, which showed a high content of aliphatic substituents (a more branched structure). In addition, the low polydispersity value (close to 1), indicates that most lignin fragments have similar molecular masses [20]

3.2.5. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)

The thermal stability of *C. tapia* lignin was investigated by TGA and DTG. Fig. 5A shows the thermogravimetric (TGA) and first derivative of mass loss (DTG) curves for *C. tapia* lignin, while Fig. 5B shows the DSC curve. Fig. 5A shows three events during *C. tapia* lignin degradation. The first event, observed up to 100 °C, reflected the loss of water with a loss of mass of ~3%. The second event, which occurred between 150 °C and 400 °C, was associated with the decomposition of compounds present in the normal lignin structure, such as fragments of carbohydrates, aliphatic alcohols, esters and acids, all of low molecular mass [59,60]. In the third event, between 400 °C and 650 °C, there was a 63.7% loss of mass that was related to the breaking of bonds between phenolic hydroxyls, carboxylic groups and benzyl hydroxyls, in addition to the degradation or condensation of aromatic rings [59,60].

The maximum peak seen in the DTG curve of *C. tapia* lignin represented the maximum temperature of thermal decomposition and corresponded to 350 °C. Watkins et al. [59], who studied lignins from wheat straw, flax fiber, alfalfa and pine straw noted that thermal decomposition occurred at 320–340 °C for all samples, with the order of thermal stability being lignin extracted from wheat straw > flax fiber > alfalfa > pine straw. Nadji et al. [61] noted that the thermal stability of alpha grass (*Stipa tenacíssima* L.) lignin isolated by different extraction methods (soda and dioxane) varied according to the method used, with thermal degradation occurring at 456.3, 443.8 and 420.8 °C, respectively. Wang et al. [62] extracted lignins from *Fraxinus mandschurica* and *Pinus sylvestris* var. Mongolian and observed degradation temperatures in the range of 380–400 °C. These differences in

Table 5
Relative distribution of the signal areas in the chemical shift regions (ppm) of the ^{1}H NMR spectrum of acetylated *C. tapia* lignin.

Range δ (ppm)	Attribution: proton type	Area	%
9.7–7.7	Aromatic protons ortho to carbonyl groups Protons formylate in cinnamaldehyde and benzaldehyde substructures	0.010	0.6
7.7–7.5	Protons located ortho to a carbonyl group in benzaldehyde units	0.010	0.6
7.3–7.2	$CDCl_3$	0.005	0.3
7.2–6.7	Aromatic protons in guaiacyl units	0.012	0.7
6.7–6.4	Aromatic protons in syringolic units	0.005	0.3
6.4–5.9	Hα in β-O-4 vinyl proton units	0.01	0.6
5.9–5.3	Hα in β-5 units of phenylculmarines; β -O-4 substructures Hα	0.20	11.2
5.3–4.0	Hβ in β-O-4 (erythro form); Hα in pin- and syringorresinol Hβ in β-O-4 (tréo form); methylenic protons in cinnamyl alcohols Hγ in various structures	0.02	1.1
4.0–3.5	Methoxy protons	0.23	12.8
3.5–2.6	Aliphatic	0.10	5.6
2.6–2.3	Aromatic acetates	0.03	1.7
2.3–1.7	Aliphatic acetates	0.16	8.9
1.7–0.8	Region of non-oxygenated aliphatic protons	1.0	55.8

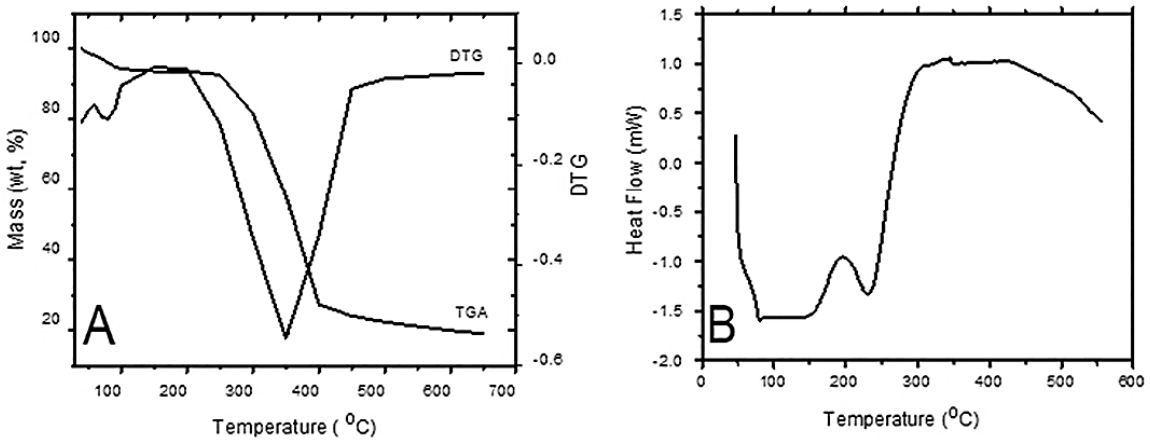

Fig. 5. TGA/DTG (A) and DSC (B) curves of *C. tapia* lignin.

degradation were associated with the chemical structure, plant species, and extraction process. As shown here, *C. tapia* lignin had high thermal stability that was conferred by its functional groups.

DSC analysis (Fig. 5B) showed the presence of an endothermic peak followed by an exothermic peak: the former peak (<100 °C) corresponded to *C. tapia* lignin dehydration while the latter (>100 °C) corresponded to *C. tapia* lignin decomposition; a similar profile was reported by Liu et al. [63], Moustaqim et al. [64] and Vallejos et al. [65] for other lignins. A maximum exothermic peak was observed at 300–450 °C and coincided with the area where the two DTG peaks were located (Fig. 5A). As with TGA, the DSC thermogram showed that *C. tapia* lignin decomposition was associated with exothermic heat fluxes.

The glass transition temperature (Tg) for *C. tapia* lignin was 98 °C, which is similar to that reported for other lignins. Tejado et al. [66] found Tg values of 100 °C and 144 °C for lignins isolated using organosolv and kraft methods. Poletto [67] reported that klason lignins from *Pinus taeda* and *Eucalyptus grandis* had higher Tg values (158 °C and 161 °C, respectively), both of which were greater than that observed here for *C. tapia* lignin. Kubo and Kadla [68] reported that commercial kraft lignin from hardwood and softwood had Tg values of 93 °C and 119 °C, respectively. Variations in the Tg values are associated with the formation of hydrogen bonds between the phenolic hydroxyl groups in the main chain of lignin [69].

Based on foregoing data it was also possible to determine the enthalpy value, which for *C. tapia* lignin was 115 J/g. Watkins et al. [59] reported that the enthalpy results for lignins from wheat straw, flax fiber, alfalfa and pine straw were 133.1 ± 2.2, 190.6 ± 8.6, 161.6 ± 1.6 and 157.9 ± 15.2 J/g, respectively.

3.3. In vitro total phenol content and antioxidant activity

The total phenol content of *C. tapia* lignin was of 189.6 ± 9.6 mg GAE/g. Cruz-Filho et al. [21] reported that lignins obtained from the cladodes of *Opuntia ficus-indica* and *Opuntia cochenillifera* by alkaline delignification had phenol contents of 36.4 ± 0.4 mg GAE/g and 87.8 ± 6.5 mg GAE/g, respectively. Aadil et al. [70] studied nine fractions of lignin from acacia wood, extracted by alkaline methods, and reported phenolic contents ranging from 73.01 ± 3.2 mg GAE/g to 393.30 ± 9.2 mg GAE/g. Santos et al. [24] reported a phenolic content of 465.9 ± 1.1 mg GAE/g for a lignin obtained from *C. erectus* leaves by alkaline delignification. Variations in the phenolic content between lignins may be associated with the extraction method, which directly affects the content of phenolic hydroxyls [70,71]. The antioxidant activity of lignin is directly related to its chemical structure, that is, it increases with the increase in the content of free hydroxyls [70–73]. And also the phenolic content is the main contributor to the antioxidant activity of lignins [72].

The ortho-methoxy substitution found in the G, S and H units of lignin contributes to the stabilization of the resonance of the phenoxyl radicals, favoring the increase in activity [70–72]. The conjugated double bonds, on the other hand, promote the additional stabilization of phenoxyl radicals through extended relocation also increasing the activity. However, lignins with a high content of conjugated carbonyl groups and aliphatic hydroxyl groups promote lower results of this activity [70–73].

Fig. 6 shows the results of the different tests of antioxidant activity in relation to the concentration of *C. tapia* lignin and it was possible to infer that the macromolecule has antioxidant action dependent on the dose, that is, as the concentration increases, there is an increase in the antioxidant activity, for the TAA and ABTS tests. Regarding the DPPH tests, the antioxidant activity showed a slight increase only at the highest concentrations and for the NO test it presented practically the same activity values for all concentrations. Table 6 presents the results of the antioxidant activity promoted by lignin by different tests in the highest concentration of the study 0.5 mg/mL.

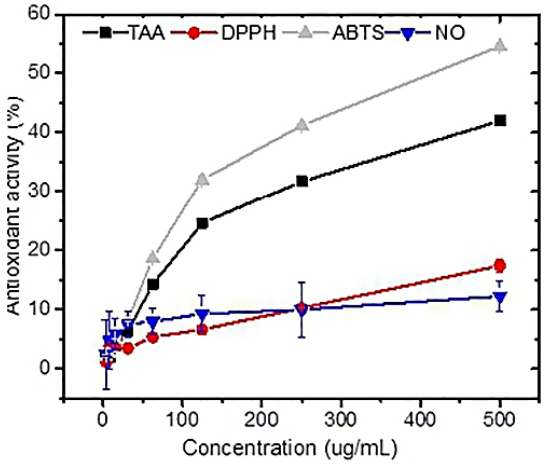

Fig. 6. Antioxidant activity promoted by *C. tapia* lignin in different tests.

Table 6
Antioxidant activity promoted by lignin from *C. tapia* leaves.

Sample	ABTS		DPPH	NO	TAA
	IC_{50} (μg/mL)	% (0.5 mg/mL)	% (0.5 mg/mL)	% (0.5 mg/mL)	% (0.5 mg/mL)
C. tapia lignin	430 ± 1.9	58.0 ± 0.01	19.7 ± 0.2	8.4 ± 1.06	18.9 ± 0.1
Ascorbic acid	90.93 ± 1.3	90.47 ± 0.34	100	*Nd*	91.05 ± 0.17

Nd - not determined; DPPH – 2,2-diphenyl-1-picrylhydrazyl radical ABTS – 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid radical; TAA – Total antioxidant activity; NO nitric oxide.

The results show that *C. tapia* lignin showed low values of antioxidant activity (<50%) when compared to ascorbic acid (>90%) for the DPPH, NO and TAA assays, and it is not possible to determine the IC_{50} (concentration that reduced the formation of radicals free by 50%) for the tests, since the content of aliphatic groups (hydrophobic) in the structure is high 55.8% (Table 5) which decrease the antioxidant activity [21,23–25,70,71].

IC_{50} determination was only possible for the ABTS assay (430 ± 1.9 μg/mL), this result was expected since the ABTS assay evaluates dual nature antioxidants with high precision, that is, the one with adapted hydrophilic groups (carboxyl, carbonyl, hydroxyl) and hydrophobic (methyl, aromatic groups), as is the case for *C. tapia* lignin [73]. In addition, *C. tapia* lignin was not able to reduce iron ions (Fe^{3+} to Fe^{2+}). Barapatre et al. [25] obtaining fractions of *Acacia nilotica* lignin by different solvents found that the fractions obtained by chloroform (CH) and n-hexane (HX) showed lower reductions in iron due to the greater amount of hydrophobic compounds.

Other authors have found different results for different lignins. Cruz-Filho et al. [21] reported that the lignins they isolated *Opuntia ficus-indica* and *Opuntia cochenillifera* had antioxidant activity, as shown by the values for DPPH (12.0 ± 0.3%; 21.1 ± 0.1%) and ABTS (26.8 ± 0.1%; 61.5 ± 0.9%). Santos et al. [24] showed that a lignin isolated from the leaves of *C. erectus* had a total antioxidant activity (TAA) of 17.92 ± 0.41%. The antioxidant activity reported by these two studies was similar to that observed for *C. tapia* lignin. It was observed that the lignins obtained by this method have higher levels of aliphatic groups, when compared to aromatic groups, which promotes low antioxidant activity. However, the results of the antioxidant activity of *C. tapia* lignin are also similar to those obtained by Huang et al. [11] the extraction of lignins from Moso bamboo (a lignin similar to that obtained in our study because it does not have sulfate groups in its chemical structure) by hydrothermal pretreatment and recovered in PS-DVB resin results from IC_{50} in a range of 460 to 910 μg/mL.

In the *Acacia nilotica* lignin [70] showed greater sequestration of the ABTS radical, when compared to *C. tapia* lignin with IC_{50} of 2.70 ± 0.1 to 3.95 ± 0.13 μg/mL. Michelin et al. [71] report an IC_{50} of 260 μg/mL and 28 μg/mL for essays DPPH and ABTS, respectively, for corn cob lignin. *Acacia nilotica* lignin [70] and corn cob [71] presented superior results of antioxidant activity when compared to *C. tapia* lignin. The authors attribute the activity to the high content of phenolic groups present in the structure of both lignins.

3.4. Cytotoxicity and immunomodulation of peripheral blood mononuclear cells (PBMC)

C. tapia lignin did not promote cytotoxic effect in the studied concentrations (2.5–80 μg/mL) in human PMBC cells *in vitro* (Fig. 7). These results indicated that the lignin in this study can be used safely in cosmetic and pharmaceutical formulations [74].

Low cytotoxicity has also been reported for other lignins. Santos et al. [24] reported that a lignin from *C. erectus* leaves was also not cytotoxic to PMBC. Cruz-Filho et al. [21] reported that two lignins from *Opuntia* were not cytotoxic to mouse splenocytes at concentrations up to 50 μg/mL. Figueiredo et al. [75] showed that carboxylated lignin nanoparticles were not cytotoxic to normal cells at concentrations up to 500 μg/mL. Ugartondo et al. [74] reported that four types of industrial lignin were not cytotoxic, and Espinoza-Acosta et al. [76], in a review of the biological effects of lignins, noted that these macromolecules are only cytotoxic at concentrations >700 μg/mL.

Lignins are known to promote immunomodulatory activity [21,23,24]. Proposing a mechanism for the immune response promoted by these macromolecules is quite complex, since the lignins present structural variations according to the origin and method of extraction [1]. However, it is known that the functional groups that stand out in the structure are: aliphatic and aromatic hydroxyls, ethers, carbonyls and methoxyls. These groups can be recognized by one or more cell receptors present on the cell surface (lignin does not cross the cell due to its molecular size) and, thus, promote different immune responses *in vitro* [77,78].

The immunomodulation assays in PBMC promoted by *C. tapia* lignin were evaluated using a lignin concentration of 10 μg/mL, since it showed the highest cell viability (95%) after 24 h. Furthermore, Melo et al. [23] and Santos et al. [24] evaluating different lignins, they found that this concentration was able to promote an immune response. Incubation of immune cells with *C. tapia* lignin stimulated the production of TNF-α (350.5 ± 35.8 pg/mL), IL-6 (283.6 ± 13.5 pg/mL) and IL-10 (154 ± 3.3 pg/mL). In addition, it did not promote a significant change in the levels of nitric oxide (NO) in the cell supernatant (Fig. 8), when compared to the control, possibly because the concentration used is not cytotoxic (NO is associated with cell death) as shown in Fig. 6.

The ability of *C. tapia* lignin to stimulate the production of TNF-α and IL-6 suggests a potential role of this lignin in the inflammatory phase of wound healing. IL-6 is important at the beginning of the healing response due to its mitogenic and proliferative action on keratinocytes (which favors healing) and its effect on neutrophils [79]. These cytokines, together with IL-1, play an important role during the inflammatory phase of healing, assisting in the recruitment of immune cells and in mediating the effects of growth factors, for example, fibroblast growth factor 7 (FGF-7), which promote epithelialization. The lignin in this study is also capable of stimulating the production of IL-10 which, according to Goulart et al. [80], can inhibit macrophage activity and decrease NO production. This decrease can also be related to the low level of production of IFN-γ observed here, since macrophages need this cytokine to stimulate the formation of NO [81].

The cytokine production profile of this work was similar to those obtained by Santos et al. [24] evaluating lignins obtained from *Conocarpus*

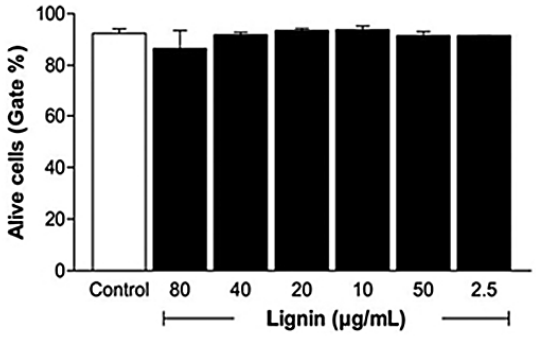

Fig. 7. Viability of human PBMC incubated with *C. tapia* lignin. The columns represent the mean ± SD ($n = 5$/independent experiments).

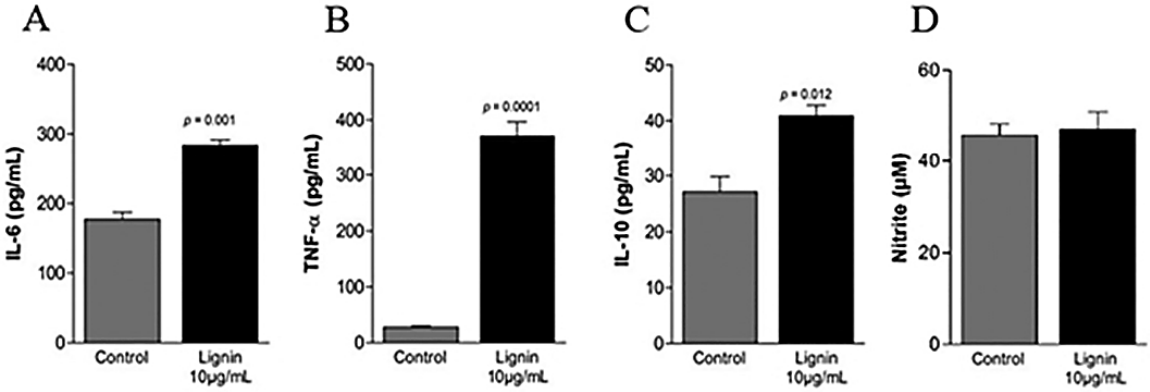

Fig. 8. Cytokine (A-C) and nitric oxide (D) concentrations in supernatants of PBMC incubated with *C. tapia* lignin for 24 h. Control cells were incubated with culture medium alone. The columns represent the mean ± SD (n = 5/independent experiments).

erectus leaves, Melo et al. [23] of lignin obtained from *Caesalpinia pulcherrima* leaves and Cruz-Filho et al. [21] lignins isolated from *O. ficus-indica* and *O. cochenillifera*. These lignins were obtained by the same extraction process (acid pretreatment followed by alkaline delignification) which generated lignins with few structural differences.

The results described here indicate that *C. tapia* lignin promotes antioxidant and immunostimulatory activities, without harming human PBMC. These results provide experimental support for the ethnopharmacological use of this plant and indicate that further studies are needed to examine the possible healing potential promoted by this macromolecule.

3.5. In vitro photoprotection by a lignin-based photoprotective cream

Lignins are considered broad-spectrum natural sunscreens capable of absorbing UVA (320 to 400 nm) and UVB (280 to 320 nm) radiation, which are harmful to man, promoting photoaging and skin cancer, inflammation, DNA damage, stress oxidative, sunburn, free radical production and immunosuppression [28,82].

According to Qian et al. [82], Lee et al. [83] and Qian et al. [28], we obtained similar results that are found in Fig. 9 and Table 7, where they show the UV absorption spectra and the results of the sun protection factor (SPF) of formulations with different concentrations of *C. tapia* lignin (1, 2, 5 and 10%).The results reveal that the increase in the concentration of lignin in the cream promoted an increase in the SPF value and that the SPF values found for the *C. tapia* lignin are close to those obtained for other lignins.

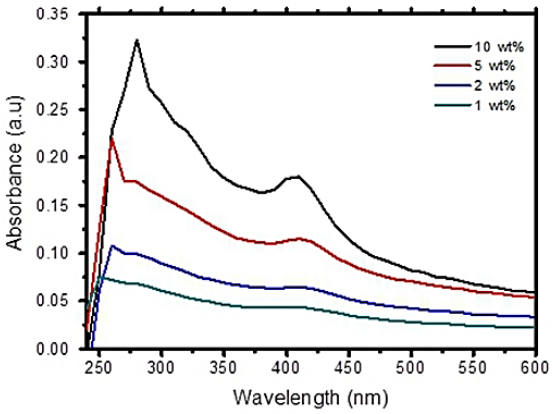

Fig. 9. UV/Vis spectrum of a cream formulation with different *C. tapia* lignin concentrations.

The photoprotective activity promoted by lignin is due to its chemical structure, that is, the presence of groups such as carbonyl, carbon-carbon and aromatic double bonds. In addition to chromophore groups, auxochromic groups such as hydroxyls and ethers promote the action of sunscreen [28,82,83]. Proposing a solar photoprotection mechanism is still a challenge, however it is known that different functional groups present in lignin are responsible for this activity [82].

In this sense, we will consider the simultaneous influence of the different functional groups present in the structure of the macromolecule. Thus, the methoxyl groups (electrode group) contribute significantly to the increase in photoprotective activity. That is, lignins with higher levels of methoxyl groups promote greater activity [82]. In addition to these groups to the aromatic regions conjugated to carbonyl groups (electron acceptor) the presence of an electron donor group in the *ortho* or *para* positions of the benzene ring, like hydroxyls (-OH) and methoxy (-OCH_3) [84–86], they also promote an increase in activity. We also highlight that the electrons located in the high-energy molecular orbital occupied (π HOMO), by absorbing UV radiation, they are excited to the low-energy empty molecular orbital (π * LUMO). When they return to their original state, they release excess energy in the form of heat or fluorescent light. [84,85]. This photoprotection phenomenon occurs through the resonance mechanism and the energy difference between the orbitals [84].

Table 7
Sun protection factor (SPF) for different concentrations of *C. tapia* lignin compared to the values obtained for different lignins.

Lignin	1%	2 ou 2.5%	5%	10%	Reference
CTL	**1.0 ± 0.0**	**1.5 ± 0.01**	**2.5 ± 0.1**	**5.1 ± 0.14**	**This study**
AL370	1.91 ± 0.09	3.68 ± 0.25	4.01 ± 0.35	6.81 ± 1.15	[82]
AL471	1.83 ± 0.09	2.76 ± 0.40	4.00 ± 0.67	6.33 ± 0.37	[82]
LS	1.46 ± 0.05	2.06 ± 0.09	3.71 ± 0.29	5.54 ± 0.96	[82]
OL	3.25 ± 0.56	5.52 ± 0.40	6.67 ± 0.25	8.66 ± 0.25	[82]
EHL	1.37 ± 0.03	2.24 ± 0.15	3.24 ± 0.28	4.20 ± 0.50	[82]
OL	1.7 ± 0.1	–	2.3 ± 0.2	3.7 ± 0.4	[83]
MWL-M	1.3 ± 0.0	–	3.2 ± 0.4	7.3 ± 0.6	[83]
MWL-P	1.2 ± 0.1	–	2.3 ± 0.1	2.6 ± 0.5	[83]
Cream-N	1.82 ± 0.13	2.74 ± 0.30	3.68 ± 0.30	5.72 ± 0.26	[28]
Cream-L	1.48 ± 0.01	1.69 ± 0.03	2.68 ± 0.17	5.33 ± 0.47	[28]

Organosolv lignin (OL), alkali lignin (95%, AL370), alkali lignin of low sulfonate content (96%, AL471), enzymatic-hydrolyzed lignin (80%, EHL), sodium lignosulfonate (LS) *Miscanthus sacchariflorus* (MWL-M), *Pinus densiflora* (MWL-P) organosolv (OL) lignin, alkaline lignin, NIVEA moisturizing cream (Creme-N) LIFE cream (Creme-L).

Thus, *C tapia* lignin shows promising results in photoprotection in conjunction with antioxidant and immunomodulatory activity. These characteristics infer that the lignin in this study can act as an adjunct to synthetic filters, which makes it possible to reduce damage to marine ecosystems and to reduce adverse effects on human health because they are not cytotoxic and promote the production of cytokines that promote healing [83,84,86].

4. Conclusion

The results of this study show that the lignin isolated from the leaves of *C. tapia* presents a structural rearrangement containing functional groups and chemical bonds (β-O-4, C-α in β-5 and β-β) characteristic of wood lignins and sugar cane bagasse. This structural rearrangement may be related to the method of obtaining which resulted in a macromolecule similar to an alkaline lignin. The obtained lignin was not cytotoxic to human PMBC *in vitro* and was able to stimulate the production of anti-inflammatory cytokines which are responsible for cell repair to external factors as in the case of solar radiation. In addition, it presented promising results of antioxidant activity in the capture of the radical ABTS, a fact that directly contributes to photoprotection, proving the ability to combat and/or prevent photodamage in the skin induced by UV radiation. These findings suggest that *C. tapia* lignin has potential pharmaceutical applications, particularly cosmetic, applied as an additive for sunscreens.

CRediT authorship contribution statement

Marcela Daniela Muniz Arruda: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing - Original draft, review & editing; **Simone da Paz Leôncio Alves**: Investigation, Validation; **Georon Ferreira de Sousa**: Investigation, Validation, Data curation, Writing - Original draft; **Guilherme Antonio de Souza Silva**: Investigation, Validation, Writing - Original draft; **Dayane Kelly Dias do Nascimento Santos**: Investigation, Validation, Data curation, Methodology, Writing – Editing; **Iranildo José da Cruz Filho**: Investigation, Validation, Data curation, Methodology, Writing - Original draft; **Maria do Carmo Alves de Lima**: Conceptualization, Methodology, Resources, Review & editing, Supervision; **George Jackson de Moraes Rocha**: Conceptualization, Writing - Review & editing, Supervision; **Ivone Antonia de Souza**: Conceptualization, Supervision, Project administration; **Cristiane Moutinho Lagos de Melo**: Conceptualization, Methodology, Formal analysis, Resources, Writing - Original draft, review & editing, Supervision, Project administration.

Declaration of competing interest

The authors have no conflict of interest with this work.

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