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Original Article

Phytochemical screening of *Cordia glabrata* (MART.) A.DC. extracts and its potential antioxidant, photoprotective, antimicrobial and antiviral activities

Triagem fitoquímica de extratos de *Cordia glabrata* (MART.) A.DC. e sua potencial atividade antioxidante, fotoprotetora, antimicrobiana e antiviral

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Abstract

Species of the genus *Cordia* have shown biological activities, such as anti-inflammatory, analgesic, antioxidant, antiviral, and antifungal activities. The species *Cordia glabrata* (MART) A.DC. Has no information concerning its phytochemical profile and possible biological activities. Thus, this study aimed to evaluate this profile in ethanolic extracts of young, adult and senescent leaves, as well as their antioxidant, photoprotective, antimicrobial, and virucidal potentials. Phytochemical analysis was performed by TLC (thin-layer chromatography) and showed the presence of flavonoids, tannins, and terpenes. The evaluation by UPLC-MS/MS (Ultra performance liquid chromatography - tandem mass spectrometer) evidenced the presence of caffeic (3.89 mgL^{-1}), *p*-cumaric (6.13 mgL^{-1}), and ferulic (0.58 mgL^{-1}) acids, whilst, in GC/MS (Gas chromatography–mass spectrometry) analysis there was a greater amount of palmitic (51.17%), stearic (20.34%), linoleic (9.62%), and miristic (8.16%) fatty acids. The DPPH (2,2-Diphenyl-1-picrylhydrazyl) and $ABTS^{+}$ (2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radicals were used to verify the potential antioxidant activity, observing a better activity for the leaf extract in the adult phenological stage: 54.63 ± 1.06 μgmL^{-1} (DPPH) and 44.21 ± 1.69 mM (ABTS). The potential photoprotective activity of the extracts was determined by spectrophotometry and the *in vitro* values of SPF (Sun Protection Factor) in young and adult leaves (5.47 and 5.41, respectively) showed values close to the minimum SPF of 6.0 required by ANVISA (Brazilian Health Regulatory Agency). It was not observed an antimicrobial activity for *Staphylococcus aureus* with a minimum inhibitory concentration of 2000 μgmL^{-1}, however the anti-herpetic assay against the *Herpes simplex* virus type 2 (HSV-2) showed a potent virucidal activity at the tested concentrations with CV_{50} value <0.195 μgmL^{-1} and a Selectivity Index ($SI = CC_{50} / CV_{50}$) greater than 448. The results obtained in this study suggest that extracts of leaves of *C. glabrata* in their adult phenological stage have potential antioxidant, photoprotective and virucidal activity, considering *in vitro* test results.

Keywords: chromatography, metabolites, HSV-2, HPLC, polyphenols.

Resumo

Espécies do gênero *Cordia* apresentam atividades biológicas, como anti-inflamatória, analgésica, antioxidante, antiviral e antifúngica. Para a espécie *Cordia glabrata* (MART) A.DC., ainda não existem informações sobre seu perfil fitoquímico e possíveis atividades biológicas, deste modo, o presente estudo teve como objetivo avaliar este perfil em extratos etanólicos de folhas jovens, adultas e senescentes, bem como o potencial antioxidante, fotoprotetor, antimicrobiano e virucida. A análise fitoquímica foi realizada por CCD (Cromatografia em Camada Delgada), mostrando a presença de flavonóides, taninos e terpenos. Na avaliação por CLAE EM/EM (Cromatografia Líquida de Ultra Eficiência acoplada a Espectrometria de Massas) foi evidenciado a presença dos ácidos caféico (3,89 mgL^{-1}), *p*-cumárico (6,13 mgL^{-1}) e ferúlico (0,58 mgL^{-1}), paralelamente, na CG/EM (Cromatografia Gasosa acoplada a Espectrometria de Massas) verificou-se maior quantidade dos ácidos graxos palmítico (51,17%), esteárico (20,34%), linoléico (9,62%) e mirístico (8,16%). Os radicais DPPH (2,2-Difenil-1-picrilhidrazil) e $ABTS^{+}$ (2′-Azino-bis (ácido 3-etilbenzotiazolina-6-sulfônico)) foram utilizados para verificar o potencial antioxidante, observando-se uma atividade superior para o extrato da folha em sua fase fenológica adulta: 54,63 ± 1,06 μgmL^{-1} (DPPH) e

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44,21 ± 1,69 mM ($ABTS^{+}$). A potencial atividade fotoprotetora dos extratos foi determinada espectrofotometricamente e os valores *in vitro* de FPS (Fator de Proteção Solar) em folhas jovens e adultas (5,47 e 5,41 respectivamente) apresentaram valores próximos ao FPS mínimo de 6,0 exigido pela ANVISA (Agência Nacional de Vigilância Sanitária). Não foi observada atividade antimicrobiana para *Staphylococcus aureus* sendo a concentração inibitória mínima de 2000 μgmL^{-1}, no entanto o ensaio anti-herpético contra o vírus *Herpes simplex* tipo 2 (HSV-2) mostrou uma potente atividade virucida nas concentrações testadas com um valor de CV_{50} <0,195 μgmL^{-1} e um Índice de Seletividade ($IS = CC_{50} / CV_{50}$) maior que 448. Os resultados obtidos neste estudo sugerem que extratos de folhas de *C. glabrata* em seu estágio fenológico adulto apresentam potencial antioxidante, fotoprotetora e virucida, considerando os resultados de testes *in vitro*.

Palavras-chave: cromatografia, metabólitos, HSV-2, CLAE, polifenóis.

1. Introduction

Plants are a stand out for providing substances with potential biological activity from their secondary metabolism (Tanase et al., 2019). Factors such as seasonality, temperature, water availability, nutrients, air pollution, mechanical stimuli, and attacks by pathogens, herbivores, and insects can influence the concentration and/or constitution of these metabolites (Botrel et al., 2010; Vizzotto et al., 2010). Furthermore, the discovery and supply of secondary metabolites with potential biological activity bring about great interest for the development of new products by the pharmaceutical, cosmetic, food, and agrochemical industries (Jaradat et al., 2018; Moreira-Araújo et al., 2019).

The species *Cordia glabrata* is a plant that belongs to the genus *Cordia* Linnaeus and the Boraginaceae Family, which is distributed from Central America to the central region of Argentina (IPNI, 1845; Vieira et al., 2015).

Some ethanolic leaf extracts of some species from this genus present biological activities, such as *Cordia verbenacea* and *Cordia curassavica* with anti-inflammatory and analgesic activity (Lameira et al., 1997; Bayeux et al., 2002), *Cordia boissieri* and *Cordia sinensis* Lam. with antioxidant activity (Al-Musayeib et al., 2011; Salazar-Aranda et al., 2011), and *Cordia dichotoma* and *Cordia verbenacea* with antimicrobial and antifungal activity (Matias et al., 2016; Pinho et al., 2012; Rahman and Akhtar 2016).

The species *Cordia glabrata* (Mart.) A.DC., popularly known as "louro-branco", "claraíba", "louro-de-mato-grosso" and "peteribi", occurring from the Piauí to the Northeast of Mato Grosso do Sul state, mostly concentrated in the Pantanal and Cerrado (Brazilian Savannah) regions of Mato Grosso - Brazil (Vieira et al., 2015; Moulin et al., 2016), there are still no reports on the species phytochemical profile, and its possible biological activities.

Thus, this work aimed to analyze the phytochemical profile and evaluate the potential antioxidant, photoprotective, virucidal and antimicrobial activity of the species *C. glabrata*.

2. Material and Methods

2.1. Plant Material obtention

The collection was carried out in an area from the Cerrado (Brazilian Savannah) of Mato Grosso - Brazil, at the Federal University of Mato Grosso – UFMT, *Campus* of Cuiabá (56°03'44.6"O 15°36'30.5"S). Four collections in one individual of *C. glabrata* were performed in the period between January and December 2019, during two seasonal periods of the region (rainy – October to April - and dry season - may to september) (Heckman, 1998), covering the entire plant phenological cycle. The botanical identification was performed at the Centro-Norte-Mato-Grossense Herbarium (CNMT) from the Federal University of Mato Grosso, *Campus* of Sinop, where the plant's and a exsiccate was stored under the registration number CNMT 7364 and code A3AE708 at Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen).

The collected plant material was sent to the Quality Control laboratory at UFMT, *Campus* of Sinop, where they were examined and selected, discarding material presenting fungi or injuries caused by insects and/or environmental factors. Later, the material was dried in a forced convection drying oven at a temperature of 40 ± 1 °C, for 48 hours. After dried, the material was ground in a mill and stored at room temperature sheltered from light.

2.2. Extracts obtention

The plant material was submitted to extraction by cold maceration with absolute ethanol (EtOHa) in the proportion of 1: 4 (m/v), at room temperature in a seven days cycle, with manual stirring every 24 hours. After the period of seven days the solvent was eliminated in a rotary evaporator, providing the crude ethanolic extracts. The extracts were weighed for extraction yield evaluation and then stored in properly closed bottles protected from light and refrigerated.

2.3. Reagents

All reagents and solvents used in the analyzes were analytical grade. Ethyl alcohol, methyl alcohol, aluminum chloride ($AlCl_3$), and sodium carbonate (Na_2CO_3) were supplied by Synth (Diadema, SP, Brazil) and the Folin-Ciocateau solution by Dinâmica (Indaiatuba, SP, Brazil). Ethyl acetate, methanol, formic acid, gallic acid, caffeic acid, *p*-cumaric acid, ferulic acid, quercetin, apigenin, kaempferol, pinocembrin, pinobanksin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), *2,2'-azino-bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS™), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox™), 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), naphthalene black, dimethyl sulfoxide (DMSO), Leibovitz-15 (L-15), fetal bovine serum (FBS), Penicillin G, streptomycin and amphotericin B were purchased from Sigma Aldrich Co. (St.

Louis, MO, USA). The Mueller Hinton M173 and M391 broths were purchased from Himedia (Mumbai, India).

2.4. Phytochemical screening by thin-layer chromatography (TLC)

Phytochemical screening was performed by thin-layer chromatography (TLC) according to Wagner and Bladt (2001), using silica gel 60 plates containing fluorescein (Chromatoplate Alugram® Xtra SIL G / UV254) as stationary phase, and different mobile phases, with different polarities. The visualization was performed using ultraviolet radiation 254-365 nm and chemical reagents for each class of metabolites (Wagner and Bladt, 2001; Simões et al., 2010).

2.5. Determination of total phenolic compounds

The determination of total phenolic compounds was performed using the Folin-Ciocalteau spectrophotometric method according to Roesler et al. (2007). The extracts were prepared in methanol (MeOH) at a concentration of 500 μgmL^{-1} and then diluted in order to obtain concentrations from 50 to 150 μgmL^{-1}. 1000 µL of each sample was transferred to a screw-cap test tube with 5 mL of distilled water, 1000 µL of the Folin-Ciocalteu reagent, and 1000 µL of a 7.5% sodium carbonate (Na_2CO_3) solution. The samples were homogenized and sheltered from light for one hour. The tests were performed in triplicate and the absorbance reading was done on a spectrophotometer at a wavelength of 750 nm. A blank test was performed to correct the absorbance measurements.

To prepare the calibration curve, gallic acid was used in concentrations from 10 to 100 μgmL^{-1}. The total phenolic values were expressed as gallic acid equivalent (milligram of gallic acid equivalent per gram of extract - $mgEAG.g^{-1}$ and microgram of gallic acid equivalent per ml of extract – μgmL^{-1}) (Neves et al., 2009; Santi et al., 2014).

2.6. Determination of total flavonoids

The determination of total flavonoids from the extracts was performed through the reaction with aluminum chloride ($AlCl_3$), and using quercetin as a standard according to the methodology described by Silva et al. (2017). The extracts were solubilized in EtOHa to a concentration of 1000 μgmL^{-1} and from these solutions, dilutions were made in EtOHa to concentrations of 50 to 150 μgmL^{-1}. From each sample, 500 to 1500 µL aliquots were transferred to 10 mL volumetric flasks, then 2 mL of a 5.0% $AlCl_3$ solution was added to each flask, and the volume was completed with EtOHa. The samples were homogenized and left to rest sheltered from light for 30 minutes, all of them performed in triplicate. The absorbance of the samples was determined in a spectrophotometer at a wavelength of 425 nm. A blank test was performed to correct the absorbance measurements and a calibration curve was prepared using quercetin as a standard, in concentrations from 3.0 to 9.0 μgmL^{-1}. Values were expressed as quercetin equivalent (milligram of quercetin equivalent per gram of extract - $mgEQ.g^{-1}$ and microgram of quercetin equivalent per ml of extract – μgmL^{-1}).

2.7. Determination of phenolic compounds by Ultra-efficient Liquid Chromatography coupled to Mass Spectrometry (UPLC-MS/MS)

The analysis was performed by a UPLC-MS/MS system (Agilent 1290 Infinity with triple quadrupole Agilent 6460) in multiple reaction monitoring (MRM) mode. The ethanolic adult leaves extracts were dissolved in ethyl acetate and diluted to a concentration of 2.0 $mgmL^{-1}$. The UPLC-MS/MS analysis used the C-18 silica gel column as stationary phase (Zorbax Eclipse AAA 4.6 x 150 mm, 3.5 µm particle size, Agilent, Santa Clara – CA, United States). The mobile phase consisted of the solvents: A (MeOH + 0.1% formic acid), and B (Milli-Q water + 0.1% formic acid), with an injection flow of 0.3 $mL.min^{-1}$. The initial elution gradient consisted of 60% (A) and 40% (B), increasing linearly to 90% of A for 10 minutes, 90% of A in isocratic mode for 3 minutes, decreasing linearly to 60% of A during 2 minutes and ending with 60% of A isocratically for 4 minutes. The extracts were detected by mass spectrometry using electrospray ionization in the negative mode. The gradient elution method of the sample had a source temperature of 300 °C and a desolvation temperature of 250 °C. The results were compared to the standards (gallic acid, caffeic acid, *p*-cumaric acid, ferulic acid, quercetin, apigenin, kaempferol, pinocembrin, pinobanksina).

2.8. Determination of fatty acids by Gas Chromatography coupled to Mass Spectrometry (GC-MS)

The *C. glabrata* leaves extract was esterified according to Jham et al. (1982). The fatty acid composition of the extract was determined in a gas chromatograph (Shimadzu - GCMS-QP2010 Ultra) connected to a mass spectrometer (QP2010 Ultra), using helium as the carrier gas (1.0 $mLmin^{-1}$) under the following conditions: 1 µL of sample injection volume; Split 3: 1; column: HP-5MS (30m x 0,25mm) packed with (5%-phenyl)-methylpolysiloxane; MS source: 230 ° C; Quad MS: 150 ° C; gradient: 140 ° C (2 minutes), 4 ° $Cmin^{-1}$ to 180 °C, 0.5 °$Cmin^{-1}$ to 200 ° C, 5 ° $Cmin^{-1}$ to 250 ° C (3 minutes); Full time: 70 minutes. Fatty acids were identified based on the retention time of the standard (Lipid Standards Sigma-Aldrich: FAMEs mixtures C8:0 – C22:0), injected under the same conditions.

2.9. Evaluation of potential antioxidant activity by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method

The potential antioxidant activity by the DPPH radical scavenging method was carried out as described by Rufino et al. (2007) and Pires et al. (2017). The extract solutions were prepared at a concentration of 4000 μgmL^{-1} and diluted with ethanol to concentrations of 8.0 to 400 μgmL^{-1}. 1000 µL of each sample was transferred to screw-cap test tubes and 3 ml of the DPPH radical solution was added. The samples were homogenized and, after a period of 30 minutes in the absence of light, the absorbance was read at a wavelength of 517 nm. The tests were performed in triplicate and a blank test was performed to correct the absorbance measurements. The results were expressed as EC_{50}, which is the extract concentration needed to reduce 50% of DPPH.

2.10. Evaluation of the potential antioxidant activity by the ABTS method (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

The ABTS radical scavenging method was performed as described by Rufino et al. (2007). The extract solutions were prepared with absolute ethanol (EtOHa) at a concentration of 1000 μgmL^{-1} and then diluted with etanol to concentrations from 300 to 1000 μgmL^{-1}. 30 µL of the solutions and 3 mL of the ABTS radical solution were transferred to screw-cap test tubes, then the mixture was homogenized and left to rest sheltered from light for six minutes. All analyzes were performed in triplicate and a blank test was performed to correct the absorbance measurements. The absorbance reading was performed at a wavelength of 734 nm.

A calibration curve was prepared with Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) in concentrations of 100 to 2000 µM. The potential antioxidant activity was calculated in relation to the Trolox activity, and the results are expressed in µM of $Trolox.g^{-1}$ of leaves

2.11. In vitro determination of the SPF

The *in vitro* sun protection factor (SPF) for *C. glabrata* extracts was determined according to the protocol established by Mansur et al. (1986). The leaves extracts solutions used to determine the SPF were prepared at a concentration of 200 μgmL^{-1} in EtOHa. The equation that establishes the SPF is given by:

$$SPF = FC. \sum_{290}^{320} EE\ (\lambda).\ 2\ .\ I\ (\lambda).\ Abs\ (\lambda) \quad (1)$$

Where: CF = correction factor (equal to 10); EE (λ) = erythematogenic effect of the wavelength λ radiation; I (λ) = sunlight intensity at the wavelength λ; Abs (λ) = spectrophotometric reading of the solution absorbance at the wavelength (λ).

2.12. Evaluation of potential antimicrobial activity

Strains of *Staphylococcus aureus* (ATCC 25923) were used for the assay. The potential antimicrobial activity was performed using the broth microdilution technique following the recommendations from the Clinical and Laboratory Standards Institute - CLSI (2018, 2020). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract capable of reducing microbial growth by ≥ 90% compared to the control growth.

Microplates containing 96 wells were used to perform the assay, they were filled with 100 µL of Mueller Hinton broth containing different concentrations of the leaves extracts (62.5-2000 μgmL^{-1}) and the same volume of bacterial suspension containing 106 $CFUmL^{-1}$ in broth Mueller Hinton (after inoculation, each well had approximately 5 x 105 $CFUmL^{-1}$) was added. The microplate was incubated at 36 ± 1 ºC, aerobically for 24 hours. After incubation, the plate was homogenized and read in a spectrophotometer at 630 nm. Bacterial growth control was also performed. All tests were performed in triplicate.

2.13. Evaluation of the potential antiviral activity against Herpes simplex virus type 2 (HSV-2)

2.13.1. Preparation of the extract for analysis

The extract of *C. glabrata* was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10,000 μgmL^{-1}, stored at -20 °C and diluted in culture medium at the time of use, not exceeding the concentration of 1% DMSO.

2.13.2. Cells and viruses

Vero E6 cells were cultivated in Leibovitz-15 (L-15) medium supplemented with 5% fetal bovine serum (FBS), penicillin G (100 UmL-1), streptomycin (100 µgmL-1), and amphotericin B (25 µgmL-1). Cell cultures were maintained at 37 °C. The HSV-2 virus strain 333 (Department of Clinical Virology, University of Gotebörg, Sweden) was propagated in Vero E6 cells. The viral titer was determined by the plate reduction method (Burleson et al., 1992) and expressed through the number of plaque-forming units per ml ($PFUmL^{-1}$). All analyzes were performed in triplicate.

2.13.3. Evaluation of cytotoxicity

The cytotoxicity of the samples was determined by the [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (MTT) assay (Mosmann, 1983). Vero E6 cell suspensions containing approximately $1.8x10^5$ cells per ml were distributed in 96 well plates (100 µL per well) and incubated for 24 h at 37 ºC. After this period, the medium was removed from the plate and 200 µL of different dilutions of the extracts (100 to 0.78 μgmL^{-1}; 1: 2 ratio) were added to the cells. Cell control was performed on the same plate (200 µL medium in each well). The plates were incubated for 48 h at 37 ºC. After this period, the medium was replaced by 50 µL of MTT (1 $mgmL^{-1}$ in L-15 medium) and the plates were incubated for 4 hours. The MTT solution was removed, 100 µL of DMSO was added to each well, the plate was shaken for 10 min on a plate shaker and the absorbance was read at 492 nm. The extract concentration value capable of reducing cell viability by 50% (CC_{50}) in relation to cell control was calculated by linear regression analysis.

2.13.4. In vitro screening for anti-HSV-2 activity

The suspensions of Vero E6 cells (± $1.8x10^5$ cells per ml) were distributed in 96 well plates and incubated for 24 h at 37 ºC. After this period, the medium was removed from the plate and the monolayer of Vero E6 cells was infected with the HSV-2 virus (0.05 MOI) and simultaneously treated with four different concentrations of the extract for 48 h. Acyclovir (15 µM) was used as a positive control for inhibition of HSV-2 strain 333. The same method described above was used to assess cell viability with MTT. The inhibition percentages were calculated using the formula [(A-B) / (C-B) x100], where A, B and C indicate the extract, the viral and the cellular control absorbances, respectively.

2.13.5. Evaluation of the virucidal activity

Mixtures of 200 µl of different concentrations of the extracts (50 to 0.195 μgmL^{-1}) and 200 µl of the viral

suspension (25-50 PFU) were mixed and incubated at 37 ºC for 30 minutes. After this time, the residual infectivity of these suspensions was determined by the plate reduction assay. Therefore, Vero E6 cells ($1.8x10^5$ cells per mL) cultivated in 24-well plates until confluence were infected with 400 µL of the viral suspension and incubated for 1 h at 37 ºC. After removing the supernatant, 1 ml of the 1.5% carboxymethyl cellulose solution in L-15 medium was added. Untreated wells were used as infection controls. The plates were incubated for 2 days at 37 ºC. After this period, the medium was removed and the cells were stained with 600 µL of 1% naphthalene black for 1 hour under mechanical stirring at room temperature. The percentages of residual infectivity of the extract were calculated in relation to the infection control. From these data, it was possible to calculate the concentration that reduces viral infectivity by 50% (CV_{50}) by linear regression analysis.

2.14. Statistical analysis

The determination of total phenolic and flavonoid compounds, the antioxidant activity analyzes by the $ABTS^+$ and DPPH methods, and the virucide and antimicrobial methods were compared by analysis of variance (ANOVA). Significant differences between the mean values were determined by Tukey's multiple comparison test with 95% significance ($p < 0.05$) and the results were presented with the mean ± standard deviation using the OriginPro program, v 8 (OriginLab©). The correlation coefficient was determined by the Pearson test, using the Microsoft Excel® 2016 program.

3. Results

Two collections were carried out during the rainy season, with the presence of young and adult leaves, and only one in the dry season, during the presence of senescent leaves, before seasonal leaf fall and flower development. Therefore, the phenological stages of the plant are young and adult leaves during the rainy season, senescent leaves and flower blossoming in the dry season.

The yield values were obtained from the relation between the crude leaves extracts and the previously weighed masses of the plant materials. The young leaves extract showed the lowest yield, followed by an increase for adult leaves and a subsequent decrease in senescent leaves (Table 1).

The phytochemical screening of leaves extracts as shown in Table 2 showed the presence of metabolites derived from phenolic compounds, such as flavonoids, tannins, and terpenes.

In the determination of phenolic compounds tests, the adult leaves extract showed a higher concentration (89.11 ± 0.46 $mgEAG.g^{-1}$) followed by senescent leaves (79.07 ± 0.60 $mgEAG.g^{-1}$) and young leaves (65.11 ± 1.52 $mgEAG.g^{-1}$). The same tendency was observed for the flavonoids, that is, higher content in the adult leaves extract (15.37 ± 0.11 $mgEQ.g^{-1}$), followed by young leaves (14.30 ± 0.11 $mgEQ.g^{-1}$) and senescent leaves (12.03 ± 0.01 $mgEQ.g^{-1}$) (Table 3).

In order to quantify some phenolic compounds and fatty acids present in the adult leaves of *C. glabrata* (because it is the phenological stage with the highest content of these metabolites), liquid and gas chromatography (UPLC-MS/MS and GC/MS) were performed. The values obtained by UPLC-MS/MS for the nine compounds analised are shown in Table 4.

The concentrations of gallic acid, quercetin, apigenin, kaempferol, pinocembrin, and pinobanksin were below the detection limit of the method.

Table 5 shows the fatty acids determined for *C. glabrata* by GC/MS, where there was a greater abundance of palmitic acid, comprising 51.17% of the total, followed by stearic acid (20.34%), linoleic acid (9.62%) and myristic acid (8.16%).

The potential antioxidant activity of the extracts was assessed using the DPPH and $ABTS^+$ radical scavenging methods and results are shown in Table 6. For the DPPH method, the ethanolic extract of adult leaves EC_{50} values showed greater antioxidant potential (49.79 ± 0.47 μgmL^{-1}), followed by young leaves (54.63 ± 1.06 μgmL^{-1}) and senescent leaves (58.09 ± 0.39 μgmL^{-1}). For the $ABTS^+$ radical scavenging method, the young leaves extract showed less potential antioxidant activity (44.21 ± 1.69 mM) when compared to the other leaves phenophases. The extracts of adult leaves (55.40 ± 0.616 mM) and senescent leaves (57.93 ± 3.56 mM) showed no statistical difference between them ($p < 0.05$).

When the Pearson's correlation was performed, a strong positive correlation was found between the amount of phenolic compounds and flavonoids. The evaluation by the DPPH method showed a strong negative correlation between the method and the concentration of phenolic compounds, as well as for flavonoids. As for the relation between phenolic compounds and the $ABTS^+$ method, the correlation was positive and moderate, and between the $ABTS^+$ method and the concentration of flavonoids the correlation was positive and weak. The moderate negative correlation observed between the $ABTS^+$ and DPPH methods (Table 7).

The SPF values found for the extracts from Equation 1 showed a significant difference by the Tukey test ($p < 0.05$), presenting an SPF of 5.47 ± 0.00 for young leaves, 5.41 ± 0.00 for adults, and 4.10 ± 0.00 for the senescent leaves.

Several species of the *Cordia* genus have promising biological activities, such as antimicrobial and antiviral (Matias et al., 2015). For the potential antimicrobial activity, no significant activity was observed for extracts of the *C. glabrata* adult leaves. The value for the minimum inhibitory concentration (MIC) of the analyzed extract was 2000 μgmL^{-1} for the *Staphylococcus aureus* strain that was tested (Table 8).

The analysis of the potential anti-HSV-2 activity of the *C. glabrata* adult leaves extract through the MTT assay showed a cytotoxicity of CC_{50} = 87.43 ± 17.26 μgmL^{-1} against Vero E6 cells, and presented 29.98 ± 5.82% of replication inhibition of the HSV-2 strain 333, at the maximum non-toxic concentration of 50 μgmL^{-1} by the same assay (Table 9).

In addition, the adult leaves extract was subjected to evaluation of virucidal activity, through the plate reduction assay. The adult leave extract showed a potent virucidal action at the tested concentrations, with a CV_{50} value <0.195 μgmL^{-1} and a Selectivity Index ($SI = CC_{50} / CV_{50}$) greater than 448 (Figure 1).

Table 1. *C. glabrata* crude extract yield in different phenological stages.

	EtOHa extracts		
	YL	**AL**	**SL**
Plant material weight (g)	50	50	50
Extract weight (g)	0.574	0.681	0.577
Yield (%)	1.148[a]	1.362[b]	1.154[c]

(EtOHa) absolute ethanol; (YL) Young Leaves; (AL) Adult Leaves; (SL) Senescent Leaves. Values with different letters demonstrate statistical diffrence ($p < 0.05$).

Table 2. Phytochemical screening of the *C. glabrata extracts.*

Phytochemical classes	**Reactions**	**EtOHa extracts**		
		YL	**AL**	**SL**
Flavonoids	TLC	+	+	**+**
Tannins	TLC	+	+	**+**
Terpenes	TLC	+	+	**+**
Coumarins	TLC	-	-	**-**
	KOH	-	-	**-**
Saponines	TLC	-	-	**-**
	Foam index	-	-	**-**
	TLC	-	-	**-**
	Dragendorff	-	-	**-**
Alkaloids	Mayer	-	-	**-**
	Bertrand	-	-	**-**
	Bouchardat	-	-	**-**
Anthraquinones	Borntraeger	-	-	**-**

(EtOHa) absolute ethanol; (YL) Young Leaves; (AL) Adult Leaves; (SL) Senescent Leaves; (+) Presence of the metabolite; (-) Absence of the metabolite.

Table 3. Phenolic compounds and total flavonoids concentrations in the ethanolic extracts of *C. glabrata* leaves and flowers.

Codes	**Phenols**	**Phenols**	**Flavonoids**	**Flavonoids**
	(mgEAG.g^{-1})	**(µgmL^{-1})***	**(mgEQ.g^{-1})**	**(µgmL^{-1})****
YL	91.16 ± 1.39[a]	22.79 ± 0.35[d]	6.81 ± 0.03[g]	10.22 ± 0.04[j]
AL	104.17 ± 0.74[b]	26.04 ± 0.18[e]	6.86 ± 0.01[h]	10.30 ± 0.02[k]
SL	68.51 ± 1.95[c]	17.13 ± 0.48[f]	3.90 ± 0.00[i]	5.85 ± 0.00[l]

(mgEAG.g^{-1}) milligram of gallic acid equivalent per gram of extract; (mgEQ.g^{-1}) milligram of quercetin equivalent per gram of extract; (µgmL^{-1}); *microgram of gallic acid equivalent per mL of extract; (µgmL^{-1}); **microgram of quercetin equivalent per mL of extract; (YL) Young Leaves; (AL) Adult Leaves; (SL) Senescent Leaves. Values with different letters demonstrate statistical diffrence ($p < 0.05$).

Table 4. UPLC-MS/MS quantification of phenolic compounds in adult leaves of *C. glabrata.*

Peak No.	**Compound**	**RT (min.)**	**Transitions Ions**	**Amount in the adult leav extract (mg L^{-1})**
1	Gallic acid[a]	4.8	168.7 - 124.7	<L.Q
2	Caffeic acid[a]	5.7	178.7 - 134.7	3.89
3	*P*-cumaric acid[a]	6.5	162.7 - 118.7	6.13
4	Ferulic acid[a]	7.8	192.9 - 133.9	0.58
5	Quercetin[a]	9.5	284.9 – 116.8	<L.Q
6	Kaempferol[a]	11	284.9 – 92.9	<L.Q
7	Apigenin[a]	11.5	269 – 116.7	<L.Q
8	Pinobanksin[a]	10	272.7 – 226.8	<L.Q
9	Pinocembrin[a]	13.5	256.7 – 152.9	<L.Q

[a]Based on standart; (L.Q) Value below the quantification limit of the method (0.1 mgL^{-1})

Table 5. Fatty acid quantification in the *C. glabrata* ethanolic extract.

	FATTY ACID	Adult Leaves	
		Area	%
1	Capric acid	15681	0.12
2	Lauric acid	50763	0.39
3	Myristic acid	1050174	8.16
4	Palmitoleic acid	111659	0.87
5	Palmitic acid	6582208	51.17
6	Linoleic acid	1237771	9.62
7	Oleic acid	362694	2.82
8	Stearic acid	2615964	20.34
9	Arachidic acid	399612	3.11
10	Erucic acid	297	N.D.
11	Beenic acid	524	N.D.
12	Lignoceric acid	436882	3.40
	Total	**12864229**	**100**

(N.D.) Not detected.

Table 6. *C. glabrata* ethanolic extracts antioxidants activity through DPPH and ABTS^{+} radical scavenging methods.

Codes	EC_{50} (μgmL^{-1})	ABTS (mM)
YL	54.63 ± 1.06^{a}	44.21 ± 1.69^{a}
AL	49.79 ± 0.47^{b}	55.40 ± 0.616^{b}
SL	58.09 ± 0.39^{c}	57.93 ± 3.56^{b}

EC_{50} (μgmL^{-1}) expressed in μg of extract /mL of DPPH; (mM) Values expressed in millimolar of trolox per g of extract; (YL) Young Leaves; (AL) Adult Leaves; (SL) Senescent Leaves. Values with different letters demonstrate statistical diffrence ($p < 0.05$).

Table 7. Correlation between the presence of phenolic compounds and the antioxidant activity determined through the Pearson's correlation coefficient.

	Phenols	Flavonoids	DPPH	ABTS
	(μgmL^{-1})	(μgmL^{-1})	(μgmL^{-1})	(mM)
Phenols (μgmL^{-1})	**1**			
Flavonoids (μgmL^{-1})	0.9358	**1**		
DPPH (μgmL^{-1})	-0.9468	-0.8124	**1**	
ABTS (mM)	0.5499	0.3394	-0.5118	**1**

Table 8. Growth inhibition of *Staphylococcus aureus* by ethanolic extracts obtained from leaves of *Cordia glabrata.*

Sample	Growth inhibition (%)						
	62.5	125	250	500	1000	2000	MIC
	μgmL^{-1}	μgmL^{-1}	μgmL^{-1}	μgmL^{-1}	μgmL^{-1}	μgmL^{-1}	μgmL^{-1}
AL	5.7 ± 0.0	10.9 ± 0.0	12.3 ± 2.3	14.3 ± 0.3	33.8 ± 3.4	100.0 ± 5.2	2000

(AL) Adult Leaves extract; (MIC) Minimum inhibitory concentration.

Table 9. Cytotoxicity and potential antiviral and virucidal activity of *C. glabrata* adult leaves extract against *Herpes Simplex* Virus type 2 (HSV-2) strain 333.

Sample	CC_{50}	% of inhibition*	CV_{50}
	μgmL^{-1}		μgmL^{-1}
AL	87.43 ± 17.26	29.98 ± 5.82	< 0.195
Aciclovir^{a} (15 μM)	-	100	-

CC_{50} = 50% cytotoxic concentration; CV_{50} = 50% virucidal concentration; (*) maximum percentage of inhibition of HSV-2 viral replication at the maximum non-toxic concentration (50 μgmL^{-1}) tested; (^{a}) positive control of HSV-2 replication inhibition.

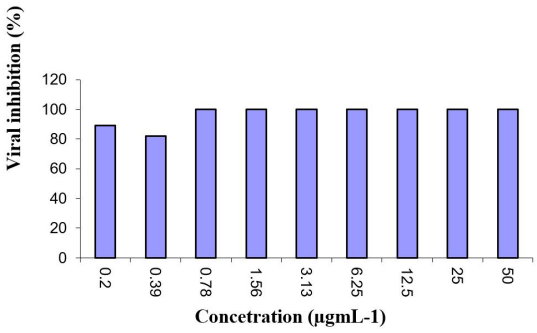

Figure 1. Results of the evaluation of the *C. glabrata* extract virucidal activity against HSV-2 strain 333 by the plate reduction assay. Percentages of viral inhibition were determined in comparison to controls.

4. Discussion

The species *C. glabrata* is abundant in the Cerrado (Brazilian Savannah) region of Mato Grosso and presents different phenophases: during the rain season, young and adult leaves are observed, and in the dry season, the presence of senescent leaves. In the dry season, the presence of flowers can be observed (Lorenzi, 1992; Melo and Lyra-Lemos, 2008).

The yield difference observed between the different leaves can be explained by the variation, mainly, in the content of chlorophyll and other metabolites present in the different phenophases of the plant material, since during the phenological development of a plant the concentration of metabolites in its tissues is variable during each period, as it happens with chlorophyll, which according to Streit et al. (2005) are found in nature in approximate proportions of 3:1. In the development of a plant, lower chlorophyll levels are observed in young leaves with a progressive increase during its maturing process, reaching a plateau during its adult phase. With the senescence of the leaves, chlorophyll and other metabolites, such as carotenoids, are reabsorbed so that biological recycling can happen, directing these metabolites to other plant tissues. (Maia and Piedade, 2002; Sousa et al., 2015).

The metabolites observed in the phytochemical screening of *C. glabrata* corroborate with reports from other authors that evidenced the presence of these metabolites in species from the *Cordia* genus (Matias et al., 2015). Phenolic compounds are commonly found in the plantae kingdom and are important due to their different biological activities such as antioxidant, anti-inflammatory, antimicrobial, antiviral and photoprotective (Nicácio et al., 2017).

Prabu et al. (2018) worked with methanolic extracts from *Cordia diffusa* leaves and found values of 5.03 ± 0.50 µgmL^{-1} for phenolic compounds and 5.65 ± 0.43 µgmL^{-1} for flavonoids, while Santi et al. (2014) found values of 79.48 ± 0.63 mgEAG g^{-1} for phenols and 65.03 ± 4.59 mgEQ g^{-1} for flavonoids in ethanolic extracts of *C. verbenacea* leaves. The results for *C. glabrata* were higher, with values from 17.13 to 26.04 µgmL^{-1} of phenols and 5.85 to 10.30 µgmL^{-1} for flavonoids. For phenols, the values obtained were 68.51 to 104.17, in mgEAG g^{-1} and 3.90 to 6.86 of flavonoids, in mgEQ g^{-1}.

The quantified metabolites in *C. glabrata* were the ferulic acid, *p*-cumáric acid and caffeic acid, as already reported in other species of the genus *Cordia*, such as gallic and caffeic acid found in *C. verbenacea* (Matias et al., 2015), ferulic acid in *C. diffusa*, (Prabu et al., 2018), quercetin and apigenin in *C. dichotoma*, (Rahman and Akhtar, 2016) and kaempferol in *C. sinensis* (Al-Musayeib et al., 2011). According to some authors, it is suggested that gallic acid, ferulic acid, *p*-cumaric acid, caffeic acid, quercetin, and apigenin have a direct role in antioxidant, antiviral, antimicrobial, and photoprotective processes (Nicácio et al., 2017).

Greater amounts of palmitic acid, stearic acid, linoleic acid and myristic acid found in *C. glabrata* agrees with other authors who point out the presence of fatty acids in the genus *Cordia* as already described by Adeosun et al. (2015) who identified palmitic, oleic, linoleic, stearic, myristic, and palmitoleic acids, for the species *Cordia sebestena*, *Cordia ecalyculata*, *Cordia myxa* and *Cordia sellowiana*. The presence of fatty acids in the composition of plant extracts can be useful for the pharmaceutical, cosmetic, and food industries, among others, since several fatty acids reportedly present potential antioxidant, anti-inflammatory, and antidepressant activities, besides helping to improve essential skin conditions (Mohd-Nasir and Mohd-Setapar, 2018).

During the different phenophases of the leaves, there was significant variation in the EC_{50} values provided by the DPPH radical scavenging method. The greater potential antioxidant activity found in the extract of adult leaves can be explained by the accumulation of metabolites produced during the growth process of this organ, with a subsequent decrease in senescent leaves, where the accumulated metabolites are reabsorbed by the plant to be reused (Maia and Piedade, 2002). From the DPPH and $ABTS^+$ data, it was possible to verify a tendency between the results of the methods, that is, even if different mechanisms of analysis were presented, the values obtained showed the same profile of potential antioxidant activity for the analyzed extracts (Gaber et al., 2021). The DPPH method has proven to be easier, of high precision and reproduction for the analysis of plant extracts and isolated compounds when compared to the $ABTS^+$ method, besides that, the radical DPPH reacts emphatically with polyphenols and $ABTS^+$ with a greater amount of phenolic compounds (Mareček et al., 2017).

Santi et al. (2014) performed the DPPH radical scavenging method with *C. verbeneacea* and found an EC_{50} value of 316.70 ± 23.16 µgmL^{-1}, revealing a lower potential antioxidant activity when compared with the data described in this study for the species *C. glabrata*, which showed EC_{50} values between 49.79 to 58.09 µgmL^{-1}.

Thus, when comparing *C. glabrata* with other species of the genus, its potential antioxidant activity *in vitro* is higher.

When Pearson's correlation was performed between the results from phenolic compounds, flavonoids, and potential antioxidant activity by the DPPH and $ABTS^+$ methods assays, a direct correlation was observed between the concentration of phenolic and flavonoid compounds, and between the $ABTS^+$ method and the phenolic and

flavonoid compounds. An indirect correlation was found for the antioxidant activity by the DPPH method and the concentration of phenolic compounds, between DPPH and flavonoids, and between the two methods of antioxidant evaluation. The Strong positive correlation by Pearson's method found between the amount of phenolic compounds and flavonoids, shows that the increase in the concentration of phenolic compounds, directly reflects in the concentration increase of flavonoids. Unlike the DPPH free radical, $ABTS^+$ reacts with a greater amount of phenolic compounds, showing that the greater the spectrum of phenolic compounds, the greater the antioxidant activity observed. The moderate negative correlation observed between the $ABTS^+$ and DPPH methods pointed out the existence of an inversely proportional relationship between both tests.

The presence of metabolites in plant extracts with potential antioxidant activity arouses interest in research due to their photoprotective activity, since many of the metabolites that perform antioxidant activity are also responsible for protecting plant tissue against UV radiation (Lefahal et al., 2018; Nunes et al., 2018).

According to Brazilian law, Collegiate Board Resolution (RDC) Nº 30, of June 1, 2012 (Brasil, 2012), for a product to be able to play a photoprotective role, the SPF found must be equal to or greater than 6.0. Although the extracts did not have the minimum SPF value required by the Brazilian resolution to be considered as having a potential photoprotective effect, they can be associated with synthetic compounds, in order to promote a synergistic action and consequently increase the photoprotective activity, as well as decreasing possible unwanted effects of synthetic products.

The results obtained for an antimicrobial analysis, corroborate those presented by Bianco et al. (2017) which revealed that the extract of *C. glabrata* did not show antimicrobial activity against gram-positive microorganisms *Streptococcus mutans* and *Streptococcus sobrinus*. Gaetti-Jardim Junior et al. (2009) also found no antimicrobial activity for extracts of *C. glabrata* against strains of gram-negative (*Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia* and *Pseudomonas aeruginosa*) and gram-positive (*Enterococcus faecalis* and *Streptococcus mutans) microorganisms*.

The virucidal activity observed can be attributed mainly to phenolic and flavonoid compounds present in the extract (Özçelik et al., 2011). Acoording to Peter et al. (2017) caffeic acid and gallic acid present in propolis extracts have antiviral action, and Hochheim et al. (2019) when evaluating fractions enriched in phenolic and flavonoid compounds, have identified a potent anti-HSV activity, with evident virucidal effect. The direct inactivation of the viral particles (virucidal action) presented by the *C. glabrata* extract may be due to the denaturation of the viral capsid proteins of the viral envelope, which may be necessary for the adsorption of the HSV-2 virus in the cellular receptors and penetration into the host cell, as well as a possible damage to the structure of the virion (Suazo et al., 2015; Álvarez et al., 2020).

Studies with other species from the *Cordia* genus demonstrate antiviral activity, e.g. *Cordia spinescens* (Matsuse et al., 1999) against HIV and *Cordia salicifolia* (Hayashi et al., 1990) for anti-HSV activity. Thus, the ability of the extract to reduce the infectivity of HSV-2 in concentrations lower than its CC_{50} suggests that it is a promising candidate for incorporation into nanostructured systems, providing a possibility of use of the extract as a drug with virucidal action, and/or a viral inactivating agent in topical products, detergents and cosmetics.

5. Conclusion

The studies for the determination of the phytochemical profile showed the presence of flavonoids, tannins, and terpenes, in addition to the quantification of the phenolic acids: caffeic, *p*-cumaric, and ferulic, by UPLC-MS/MS. The CG-MS analysis showed that palmitic acid is the main fatty acid present in the extract, followed by stearic acid, linoleic acid, and myristic acid. The extracts showed a potential photoprotective action and can be associated with synthetic compounds in order to reduce these compounds, which are often responsible for allergenic processes.

The adult leaves extracts obtained from *C. glabrata* had superior biological activities *in vitro* (antioxidant and virucide) when compared to other species from the same genus. Thus, the data revealed in this article show promise for the isolation of *C. glabrata* compounds in order to use them in cosmetic and/or pharmaceutical products in their isolated form or as a standardized crude extract, in addition to adding value to the flora of the Cerrado (Brazilian Savannah) region of Mato Grosso.

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