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Original Article

Incorporation of *Cordia glabrata* (Mart.) A.DC. extract in microemulsions and their potential antioxidant, photoprotective and virucidal activities

Incorporação de extratos de *Cordia glabrata* (Mart.) A.DC. em microemulsões e suas potenciais atividades antioxidante, fotoprotetora e virucida

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Abstract

Extracts of species from the *Cordia* genus have been reported with potential biological activities, such as antioxidant, antimicrobial, antiviral, and antiparasitic. The aim of this study was to develop microemulsions containing ethanolic extracts of *C. glabrata* leaves and to evaluate their stability and biological activities. The five developed microemulsions presented physicochemical stability and presented Newtonian behavior when submitted to rheological analysis. The diameter values of the globules ranged between 225.74 and 273.33 nm and the zeta potential of the formulations remained between -22.40 and -25.08. All phenolic acids of the extract, quantified by HPLC, showed consistency after being microemulsified. The EC_{50} values for the antioxidant activity by the DPPH scavenging method ranged between 38.13 and 45.54 μgmL^{-1}, and between 34.46 and 39.60 mM for the $ABTS^{+}$ scavenging method. The virucidal activity presented a CV_{50} <0.195 μgmL^{-1} and a selectivity index greater than 20. The photoprotection results ranged between 2.14 to 2.84. The study revealed stable microemulsions and potentiation of the antioxidant effect in the microemulsified extracts.

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

Resumo

Extratos de espécies do gênero *Cordia* têm sido relatados com potencial atividade biológica, como antioxidante, antimicrobiana, antiviral e antiparasitária. O objetivo deste estudo foi desenvolver microemulsões contendo extratos etanólicos de folhas de *C. glabrata* e avaliar sua estabilidade e atividades biológicas. As cinco microemulsões desenvolvidas apresentaram estabilidade físico-química e apresentaram comportamento newtoniano quando submetidas à análise reológica. Os valores de diâmetro dos glóbulos variaram entre 225,74 e 273,33 nm e o potencial zeta das formulações permaneceu entre -22,40 e -25,08. Todos os ácidos fenólicos do extrato, quantificados por CLAE, apresentaram consistência após serem microemulsionados. Os valores de EC_{50} para a atividade antioxidante pelo método de eliminação DPPH variaram entre 38,13 e 45,54 μgmL^{-1}, e entre 34,46 e 39,60 mM para o método de eliminação $ABTS^{+}$. A atividade viricida apresentou CV_{50} <0,195 μgmL^{-1} e índice de seletividade maior que 20. Os resultados da fotoproteção variaram entre 2,14 a 2,84. O estudo revelou microemulsões estáveis e potencialização do efeito antioxidante nos extratos microemulsionados.

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

1. Introduction

Distributed amongst tropical, subtropical, and temperate forests, the Boraginaceae family counts with nine different genera. Within this family, the genus *Cordia* Linnaeus comprises about 250 species distributed from Central America to Central Argentina (IPNI, 1845). Within this genus belongs the *C. glabrata* (Mart.) A.DC. species, which has its leaves and stem barks used by the population in form of infusions to combat "body aches" and rheumatic symptoms (Guarim Neto, 2006).

The use of plant extracts as a therapeutic resource is a common practice in popular medicine, however, when used in form of infusions, they do not follow a standardization

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in the concentration of the used extract. Beyond that, the extracts can be easily degraded by high temperature during the process of preparation, variations in gastrointestinal pH, and the presence of digestive enzymes (Chen et al., 2018). Therefore, the development of formulations that can circumvent these factors and guarantee the stability of plant extracts during their administration has become a major attraction (Danaei et al., 2018).

Among the nanotechnological options, nanoemulsions (NEs) and microemulsions (MEs) have proven to be versatile systems that collaborate with solubilization and stable and effective release of compounds extracted from plants, since they have a reduced particle diameter (100-500 nm), which facilitates the effective pass through the biological barriers of an organism. Because of that, these formulations stand out as promising systems for the pharmaceutical, food, and cosmetic market (Torres et al., 2019; Saneja and Panda, 2020).

MEs have the advantage of forming spontaneously, with no need for strong agitation or high temperatures. The use of adequate proportions of the oily, aqueous, and surfactant phases, guarantee the physicochemical stability of the system (Ribeiro et al., 2015; Fiori et al., 2017; Tagavifar et al., 2017).

The use of these systems containing extracts from leaves of *C. glabrata* raises interest in the assessment of their potential biological activities since extracts of other species from the same genus have already presented anti-inflammatory, analgesic, antioxidant, antiviral, and antifungal activities (Losada-Barreiro and Bravo-Díaz, 2017; Silva et al., 2018).

In previous research (Debiasi et al., 2021), the present group found that the crude ethanolic extract of *C. glabrata* has an antioxidant and virucidal activity against the *Herpes simplex virus* type 2 (HSV-2). Thus, the work aimed to develop different microemulsions containing ethanolic extracts of *C. glabrata* leaves and evaluate the physicochemical stability and biological activities of the extract in the developed systems.

2. Materials and Methods

2.1. Plant material obtention

Adult leaves of *Cordia glabrata* (Mart.) A.DC. (Boraginaceae) were collected at the Federal University of Mato Grosso - UFMT *campus* of Cuiabá - MT (56° 03'44.6 "O 15° 36'30.5" S). The botanical identification was carried out in the Centro Norte Mato-grossense (CNMT) herbarium at the Federal University of Mato Grosso, *Campus* of Sinop, where the plant's exsiccata was stored under the registration number CNMT 7364 and registered under the code A3AE708 in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen).

The collected vegetable material was dried in a forced convection drying oven at a temperature of 40 ± 1 °C, for 48 hours. After drying, the material was ground in a mill and stored at room temperature sheltered from light.

2.2. Reagents

All reagents and solvents used in the analyzes were of analytical grade and/or HPLC grade. Ethyl alcohol, 1-butanol, aluminum chloride ($AlCl_3$), and sodium carbonate (Na_2CO_3) were supplied by Synth (Diadema, SP, Brazil) and the Folin-Ciocateau solution and acetic acid by Dinâmica (Indaiatuba, SP, Brazil). Methyl alcohol, acetonitrile, gallic acid, *p*-cumaric acid, ferulic acid, caffeic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS™), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), sorbitan monooleate (Span 80™), polysorbate 80 (Tween80™), Capric/Caprylic acid Triglycerides (TACC), 3-(4,5-dimethyl-2-thiazolyl)-2bromide, 5-diphenyl-2H-tetrazolium (MTT), naphthalene black, dimethyl sulfoxide (DMSO), Leibovitz-15 (L-15), fetal bovine serum (SFB), Penicillin G, streptomycin, and amphotericin B were purchased from Sigma Aldrich Co. St. Louis, MO).

2.3. Extracts obtention

The plant material (leaves) was subjected to extraction by maceration in absolute ethanol (EtOHa) in the proportion of 1:4 (w:v), at room temperature for a period of seven days, with manual agitation every 24 hours (Simões et al., 2010). After the seven-day period, the solvent was eliminated in a rotary evaporator (803, Fisatom, Brazil), providing the ethanolic extracts.

2.4. Microemulsified systems (MEC)

2.4.1. Composition of the microemulsified systems

The microemulsified systems were developed using distilled water, caprylic/capric acid triglycerides (TACC) - hydrophilic-lipophilic balance (HLB) = 10.8; sorbitan monooleate - Span 80™ (SP) - HLB = 4.3; polysorbate 80 - Tween 80™ (TW) - HLB = 15.0; 1-butanol (BT) and ethanolic extract of *C. glabrata*.

2.4.2. Development of the microemulsified systems

Five microemulsified systems were prepared, one without extract (control) and five containing the extract of *C. glabrata* (MEC) at a concentration of 1.0% according to the methodology from Ribeiro et al. (2015). The control formulations were prepared to evaluate and be compared with the effects of the microemulsified extract.

During preparation, different proportions of H_2O / TACC / SP / TW / BT (w / w / w / w / w) were used in each MEC: 1MEC = 10: 10: 28: 44: 8, 2 MEC = 10: 15: 26.25: 41.25: 7.5, 3 MEC = 10: 25: 22.75: 35.75: 6.5, 4 MEC = 10: 30: 21: 33: 6 and 5 MEC = 10: 40: 17.5: 27.5: 5.

2.5. Physicochemical characterization

The physicochemical characterization was carried out to verify possible processes of alteration and/or degradation of the systems. The prepared MECs were analyzed after 24 h of preparation, as well as in the preliminary and accelerated stability tests.

The performed tests were: centrifugation (Q222T, Quimis, Brazil) at 3600 rpm for 30 minutes at 25 ± 1 °C, pH

determination using the pH meter (DLA-PH, Del Lab, Brazil) calibrated with pH 4.0 and 7.0 buffer solutions, electrical conductivity with conductometer (MCA-150, Tecnopon, Brazil) calibrated with a standard solution of KCl 0.1 molL^{-1}, and refractive index, obtained from the application of the sample in a refractometer (WYA-2S, Polax, Brazil) (ANVISA, 2004; Fiori et al., 2017; Torres et al., 2019).

2.6. Stability studies

The prepared MECs were divided in two groups and subjected to the preliminary stability test, by means of alternating cooling/heating cycles every 24 h, at 5 ± 1 °C and 40 ± 1 °C for 14 days (ANVISA, 2004). At the end of the cycle, the samples were left at room temperature for 24 hours to then carry out the physicochemical characterization. The MECs that showed stability were prepared again and then subjected to accelerated stability for a period of 90 days.

For the accelerated stability test, the MECs were submitted to three different temperatures (5, 25, 45 ± 1 °C) and exposed to artificial light at 25 ± 1 °C. At each 30-day interval, the formulations were kept at room temperature for 24 hours and the physicochemical parameters were evaluated (ANVISA, 2004).

2.7. Rheological characterization

The rheological analysis was performed with the MECs according to Ribeiro et al. (2015) using a compact modular rheometer (MCR 102, Anton Paar GmbH, Germany). For the tests, 600 µL of each formulation was placed on the surface of the reading plate and the data was collected with continuous control of the gap measurement using the 0.099 mm TruGap™ support. The used measuring cell was a Toolmaster™ CP 50, and precise temperature control was achieved through T-Ready™. The acquired data were treated with the Rheoplus Software V3.61. The flow and viscosity curves were displayed using established parameters considering the control of the shear stress (τ), in the range of 0–5 Pa for the upward curve and 5–0 Pa for the downward curve. These measurements were performed under isothermal conditions at 25 °C, comprising 75 readings per analysis.

2.8. Dynamic Light Scattering (DLS) and Zeta Potential (ζ)

The mean particle diameter and the polydispersity index (PDI) were evaluated by dynamic light scattering (DLS), at 25 ºC using a 4 mW He-Ne laser at a wavelength of 633 nm, with scattering angle from 25º to 90 ºC (Zetasizer Nano ZS, Malvern Instruments, United Kingdom). The Zeta Potential (ζ) was obtained by electrophoretic measurements on the same equipment. For both tests, the samples were evaluated in triplicates based on a 1:100 dilution of the MECs, based on the methodology from Ribeiro et al. (2016).

2.9. Determination of phenolic acids by High-Performance Liquid Chromatography (HPLC)

The analyzes were performed with a HPLC Ultimate 3000 (HPLC Dionex UltiMate 3000, Thermo Fisher Scientific™, USA) equipped with a manual injector (20 µL), column oven, and a UV/visible detector. The separation of phenolic acids was carried out under the following conditions: C18 reverse-phase column (250 × 4.0 mm; 5 µm particle diameter; Hypersil™ BDS, Thermo Fisher Scientific™, USA) and controlled temperature at 30 ± 1 °C. The mobile phase was composed of water:acetic acid in the proportion of 98:2, v/v (phase A) and water:acetic acid:acetonitrile in the proportion of 58:2:40, v/v/v (phase B). A constant flow of 1.0 mLmin^{-1} was used, in a binary gradient of: 0-1 min., 5% B; 2-15 min., 20% B; 15-25 min., 25% B; 25-33 min., 85% B; 33-36 min., 20% B and 36-45 min. 5% B, *stopping* at 45 minutes (Bittencourt et al., 2018; Haas et al., 2018). Detection was performed at the wavelengths of 280, 300 and 320 nm.

Standard solutions of phenolic acids (*p*-cumaric, caffeic, ferulic and gallic) were prepared in methanol at a concentration of 1000 µgmL^{-1} and then diluted in the mobile phase B, providing work solutions of 5 to 10µgmL^{-1} to obtain the calibration curve.

Known concentrations of the standards were added to the samples and quantification was performed based on the ratio of the peak areas of the sample chromatograms over the peak areas of the calibration standards. The entire system was monitored and the data was processed by the Chromeleon™ software version 7.0.

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

The potential antioxidant activity by the DPPH radical scavenging method was carried out as described by Rufino et al. (2007a) and Pires et al. (2017). MECs were prepared at a concentration of 500 µgmL^{-1} and diluted to concentrations of 8.0 to 40 µgmL^{-1}. 1000 µL of each diluted sample was transferred to screw-cap test tubes and 3 mL of the DPPH radical in methanolic 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 (T80, PG Instruments, United Kingdom). 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 the DPPH.

2.11. 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 carried out as proposed by Rufino et al. (2007b). The extracts were prepared in absolute ethanol (EtOH) at a concentration of 500 µgmL^{-1} and then diluted to concentrations of 3.0 to 10 µgmL^{-1}. 30 µL of the sample solutions and 3 mL of the ABTS^{+} radical in ethanolic solution were transferred to screw-cap test tubes, homogenized, and then 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 (T80, PG Instruments, United Kingdom).

A calibration curve was constructed 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 mM of Trolox.g^{-1} of extract.

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

2.12.1. Preparation of microemulsions (MEC) for analysis

The MECs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10000 µgmL^{-1}, stored at -20 °C and diluted in culture medium at the time of use, not exceeding the concentration of 1% of DMSO.

2.12.2. 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 analyses were performed in triplicate.

2.12.3. 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). The Vero E6 cells 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 the different MEC dilutions (50 to 0.78 µgmL^{-1}) were added to the cells. Cell control was performed on the same plate (200 µL of the medium in each well). The plates were incubated for 48 hours 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 h. Then, the MTT solution was removed, 100 µL of DMSO was added to each well, the plate was shaken for 10 minutes on a plate shaker (KJ-201 BD Orbital Shaker, Global Trade Technology, China), and the absorbance was read at 492 nm (Biolisa Reader R792, Bioclin, China). The MEC concentration value capable of reducing cell viability by 50% (CC_{50}) in relation to cell control was calculated by linear regression analysis.

2.12.4. Evaluation of the virucidal activity

Mixtures of 200 µl of different concentrations of the MEC (3.125 to 0.195 µgmL^{-1}) and 200 µl of the viral suspension (25-50 PFU) were mixed and incubated at 37 °C for 30 minutes. After this time, the residual infectivity of these suspensions was determined by the plate reduction assay. Then, Vero E6 cells ($1.8x10^5$ cells per mL) cultivated in 24-well plates until confluence were infected with 200 µL of the viral suspension + the samples in different concentrations and incubated for 1 h at 37 °C. 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 °C. 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.

2.13. In vitro determination of the SPF

The *in vitro* sun protection factor (SPF) for the MECs was determined according to the protocol established by Mansur et al. (1986), by spectrophotometry. The solutions used to determine the SPF were prepared at a concentration of 200 µgmL^{-1} in absolute ethanol and read immediately in a spectrophotometer (T80, PG Instruments, Leicester, UK). The equation that establishes the SPF is given by Equation 1:

$$FPS = CF \cdot \sum_{290}^{320} EE\ (\lambda).2.I(\lambda).Abs(\lambda) \quad (1)$$

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's absorbance at the wavelength (λ).

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 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 software, v 8 (OriginLab©). HPLC analyzes were processed with Chromeleon™ software version 7.0.

3. Results

After 24 h of preparation, the samples remained stable when conducted the evaluation of their physicochemical characteristics, as well as after the preliminary stability test (14 days of cooling/heating cycle). The results of the analyzed parameters are shown in Table 1.

New MECs were prepared and submitted to accelerated stability (90 days), the results of which are described in Table 2. All formulations showed macroscopic stability after the test, with pH values between 6.52 and 7.34, electrical conductivity between 1.23 and 4.74 µScm^{-1}, and refractive index between 1.44 and 1.46.

The rheological characteristics of the formulations were evaluated considering the flow represented at a

Table 1. Parameters evaluated in the preliminary stability test of microemulsions containing ethanolic extract of *C. glabrata*.

Tests		Formulations				
		1MEC	2MEC	3MEC	4MEC	5MEC
Centrifugation	Before	N	N	N	N	N
	After	N	N	N	N	N
Aspect	Before	TL	TL	TL	TL	TL
	After	TL	TL	TL	TL	TL
pH	Before	6.60 ± 0.00	7.28 ± 0.00	7.04 ± 0.00	7.12 ± 0.00	7.34 ± 0,00
	After	6.60 ± 0.25	7.05 ± 0.19	7.00 ± 0.12	6.60 ± 0.05	7.05 ± 0,04
Refractive index	Before	1.4542 ± 0.00	1.4543 ± 0.00	1.4464 ± 0.00	1.4504 ± 0.00	1.4485 ± 0.00
	After	1.4580 ± 0.00	1.4550 ± 0.00	1.4543 ± 0.00	1.4509 ± 0.00	1.4497 ± 0.00
Conductivity (μScm^{-1})	Before	4.46 ± 0.00	4.74 ± 0.00	3.12 ± 0.00	3.20 ± 0.00	3.92 ± 0.00
	After	7.27 ± 0.57	6.41 ± 0.79	4.25 ± 0.61	3.55 ± 1.05	5.37 ± 0.82

N: Normal; TL: Translucent Liquid. Results expressed in mean ± standard deviation (S.D.).

Table 2. Physicochemical parameters evaluated at 25 °C in the accelerated stability test of microemulsions containing ethanolic extract of *C. glabrata*.

Tests	Days	Formulations				
		1MEC	2MEC	3MEC	4MEC	5MEC
pH	0	6.60 ± 0.00^{a}	7.28 ± 0.00^{a}	7.04 ± 0.00^{a}	7.12 ± 0.00^{a}	7.34 ± 0.00^{a}
	30	6.90 ± 0.06^{b}	6.92 ± 0.10^{b}	6.85 ± 0.12^{a}	6.80 ± 0.21^{b}	6.75 ± 0.29^{b}
	60	6.72 ± 0.47^{a}	6.80 ± 0.14^{c}	6.84 ± 0.21^{b}	6.80 ± 0.25^{c}	6.65 ± 0.01^{c}
	90	6.56 ± 0.14^{a}	6.56 ± 0.06^{c}	6.63 ± 0.12^{b}	6.62 ± 0.04^{c}	6.52 ± 0.16^{c}
Electrical conductivity (μScm^{-1})	0	4.46 ± 0.00^{a}	4.74 ± 0.00^{a}	3.12 ± 0.00^{a}	2.80 ± 0.00^{a}	3.92 ± 0.00^{a}
	30	4.74 ± 0.24^{a}	3.83 ± 0.04^{b}	2.77 ± 0.26^{b}	2.78 ± 0.29^{b}	2.24 ± 0.52^{b}
	60	3.91 ± 0.46^{b}	3.55 ± 1.32^{c}	2.41 ± 1.17^{c}	2.62 ± 1.34^{c}	1.79 ± 0.57^{c}
	90	4.27 ± 1.65^{b}	3.59 ± 1.19^{c}	2.03 ± 0.20^{c}	2.86 ± 1.23^{a}	1.23 ± 0.47^{c}
Refractive index	0	1.4542 ± 0.00^{a}	1.4543 ± 0.00^{a}	1.4464 ± 0.00^{a}	1.4504 ± 0.00^{a}	1.4485 ± 0.00^{a}
	30	1.4550 ± 0.00^{a}	1.4552 ± 0.00^{a}	1.4519 ± 0.00^{b}	1.4505 ± 0.00^{a}	1.4493 ± 0.00^{a}
	60	1.4598 ± 0.00^{b}	1.4600 ± 0.00^{b}	1.4565 ± 0.00^{c}	1.4549 ± 0.00^{b}	1.4544 ± 0.00^{a,b}
	90	1.4609 ± 0.00^{b}	1.4594 ± 0.00^{b}	1.4570 ± 0.00^{c}	1.4562 ± 0.00^{c}	1.4550 ± 0.00^{b}

Values with different letters in the same column demonstrate statistical diffrence ($p < 0.05$). Results expressed in mean ± standard deviation (S.D.).

Figure 1 and viscosity in Figure 2 curves as functions of the shear rate. The flow curve begins at its origin and presents linear upward and downward behavior, indicating that all formulations behave as Newtonian fluids at 25 °C. The viscosity curves demonstrate that the viscosity values do not change with the increase in the shear rate, with values between 1.5 and 2.3 Pas.

Table 3 shows values of zeta potential (variating between -22.40 and -25.08), hydrodynamic diameter of the globules (between 225.74 and 273.33 nm) at a Figure 3, and the polydispersity index (PDI) values (between 0.257 and 0.353).

For the test of antioxidant activity, the values from the control MECs were subtracted from the MECs containing extract to verify the performance of the extract after being incorporated into the formulations.

The quantification results of the phenolic acids analysis carried out by High Performance Liquid Chromatography (HPLC) for MECs containing ethanolic extract of *C. glabrata* are shown in Table 4, with concentrations ranging from 2.65 to 3.41 mgL^{-1} for gallic acid, 2.63 to 3.10 mgL^{-1} for caffeic acid, 3.49 to 4.69 mgL^{-1} for *p*-cumaric acid, and 3.55 to 3.98 mgL^{-1} for ferulic acid.

The determination of the potential antioxidant activity by the DPPH and $ABTS^{+}$ radical scavenging methods are shown in Table 5. For the DPPH, the EC_{50} values obtained were between 38.13 ± 2.61 and 45.54 ± 1.56 μgmL^{-1}, and for the analysis with the $ABTS^{+}$ radical, the values were between 34.46 ± 1.82 and 39.60 ± 1.11 mM.

The potential virucidal activity against the *Herpes Simplex* Virus type 2 (HSV-2) through the plate reduction

assay was performed with 2 MEC and 5 MEC. The choice was based on the general stability characteristics of the MECs and on the proportion of the oily phase. Microemulsions containing extracts showed a significant difference by the Tukey test ($p < 0.05$), with a greater virucidal action when compared to microemulsions without extract, with CV_{50} values <0.195 μgmL^{-1} and a selectivity index ($SI = CC_{50}/CV_{50}$) greater than 20 (Table 6).

Figure 4 shows the percentage of virucidal activity performed by the chosen MECs compared with their respective controls (MC). It appears that between the two control groups (2 and 5MC) did not show a significant difference by the Tukey test ($p < 0.05$) in the same way as for the two microemulsion (2 and 5MEC). This significant

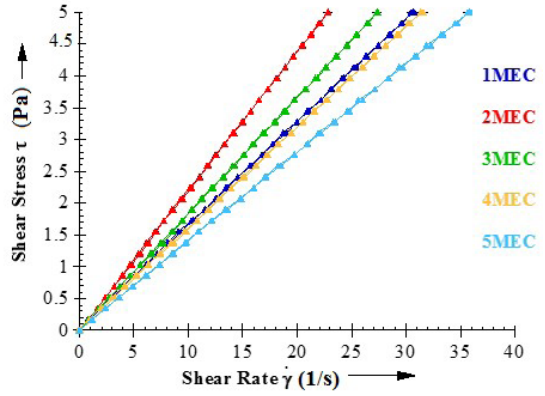

Figure 1. Flow curves of the developed MECs containing ethanolic extract of *C. glabrata* at 25 °C.

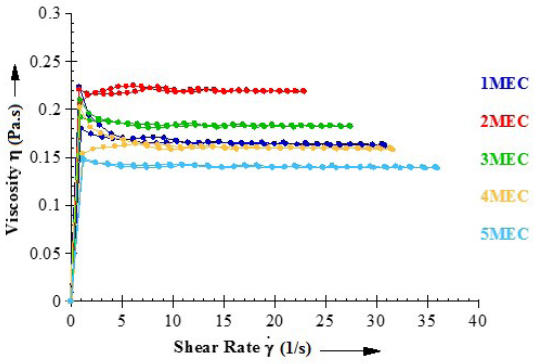

Figure 2. Viscosity curves of the developed MECs containing ethanolic extract of *C. glabrata* at 25 °C.

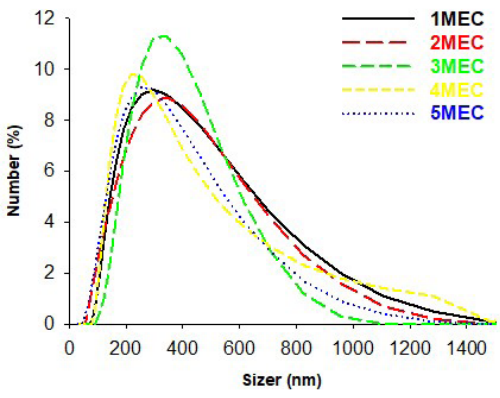

Figure 3. Globules' size distribution (nm) of the developed MECs containing ethanolic extract of *C. glabrata* at 25 °C.

Table 3. Hydrodynamic diameter and zeta potential of MECs containing *C. glabrata* extract.

Formulation	Hydrodynamic diameter (nm)	Polydispersity index (PDI)	Zeta potential (mV)
1MEC	259.37 ± 16.53	0.353 ± 0.046	-22.40 ± 1.94
2MEC	263.17 ± 21.10	0.297 ± 0.026	-24.70 ± 4.06
3MEC	273.33 ± 10.82	0.279 ± 0.088	-25.08 ± 0.65
4MEC	246.07 ± 31.16	0.330 ± 0.060	-24.45 ± 0.58
5MEC	225.74 ± 35.60	0.257 ± 0.065	-22.73 ± 1.13

Results expressed in mean ± standard deviation (S.D.).

Table 4. HPLC quantification of phenolic compounds in microemulsified and non-microemulsified *C. glabrata* extracts.

Formulation	Compound	RT (min.)	Amount (mgL^{-1})
C. glabrata extract	Gallic acid[a]	3.6	3.41
	Caffeic acid[a]	10.1	2.89
	P-cumaric acid[a]	16.3	4.69
	Ferulic acid[a]	20.2	3.98
1 MEC	Gallic acid[a]	3.6	3.29
	Caffeic acid[a]	9.8	2.86
	P-cumaric acid[a]	16.3	3.73
	Ferulic acid[a]	19.9	3.77
2 MEC	Gallic acid[a]	3.7	3.32
	Caffeic acid[a]	9.8	2.63
	P-cumaric acid[a]	16.2	3.91
	Ferulic acid[a]	19.9	3.55
3 MEC	Gallic acid[a]	3.7	2.65
	Caffeic acid[a]	10.1	2.82
	P-cumaric acid[a]	16.4	3.49
	Ferulic acid[a]	20.1	3.60
4 MEC	Gallic acid[a]	3.6	3.15
	Caffeic acid[a]	10.1	2.95
	P-cumaric acid[a]	16.4	3.82
	Ferulic acid[a]	19.9	3.73
5 MEC	Gallic acid[a]	3.6	3.31
	Caffeic acid[a]	9.8	3.10
	P-cumaric acid[a]	16.3	3.76
	Ferulic acid[a]	19.9	3.80

[a]Based on standard. RT: Retention Time.

Table 5. Antioxidants activity by DPPH and ABTS^{+} radical scavenging methods in the microemulsions containing ethanolic extracts of *C. glabrata* at 25 °C.

Formulation	DPPH EC_{50} (μgmL^{-1})	ABTS (mM)
1 MEC	45.54 ± 1.56^{a}	37.79 ± 0.98^{a,b}
2 MEC	40.42 ± 0.46^{b}	35.81 ± 1.94^{a,b}
3 MEC	42.64 ± 0.69^{c}	34.46 ± 1.82^{a}
4 MEC	38.13 ± 2.61^{d,b}	37.45 ± 0.92^{a,b}
5 MEC	44.28 ± 1.60^{e}	39.60 ± 1.11^{b}

EC_{50} (μgmL^{-1}) expressed in µg of MEC/mL of DPPH; (mM) Values expressed in millimolar of trolox per g of MEC. Values with different letters in the same column demonstrate statistical diffrence ($p < 0.05$). Results expressed in mean ± standard deviation (S.D.).

Table 6. Cytotoxicity and potential virucidal activity of MECs containing ethanolic extract of *C. glabrata* leaves against *Herpes Simplex* Virus type 2 (HSV-2) strain 333.

Formulation	*CC_{50} (μgmL^{-1})	*CV_{50} (μgmL^{-1})	SI
2 MC	7.79 ± 0.56^{a}	3.65 ± 1.20^{a}	2.13^{a}
2 MEC	5.63 ± 0.44^{b}	0.27 ± 0.15^{b}	20.77^{b}
5 MC	8.75 ± 0.04^{c}	3.72 ± 1.52^{c}	2.35^{c}
5 MEC	8.06 ± 0.04^{d}	0.40 ± 0.36^{d}	20.05^{d}

CC_{50}: 50% cytotoxic concentration; CV_{50}: 50% virucidal concentration. SI: selectivity Index; 2 and 5MC: microemulsion control; 2 and 5 MEC: microemulsion with extract. Values with different letters in the same column demonstrate statistical diffrence ($p < 0.05$). *Values represent the mean ± standard deviation (S.D.) of two independent experiments.

difference was observed only wen compared a control (MC) with your respective microemulsion containing extract (MEC).

The values of Sun Protection Factor (SPF) found in the microemulsions were 2.14 ± 0.0 for 1 MEC, 2.28 ± 0.0 for 2 MEC, 2.61 ± 0.0 for 3 MEC, 2.67 ± 0.0 for 4 MEC and 2.84 ± 0.0 for 5 MEC. All formulations showed a significant difference by the Tukey test ($p < 0.05$).

4. Discussion

The aim of this study was to develop microemulsions containing ethanolic extracts of *C. glabrata* leaves and to evaluate the stability and biological activities, in addition to the possibility of enhancing these therapeutic actions, since promising results for the crude extract have already been observed and reported by Debiasi et al. (2021).

The incorporation of the *C. glabrata* extract in the proportion of 1.0% caused the systems to remain translucent, which is indicative of preservation of the MEC's characteristics. Microemulsions containing plant extracts can favor the stability and the delivery of active substances through the skin membranes due to the small size of the formed droplets, making it more advantageous when

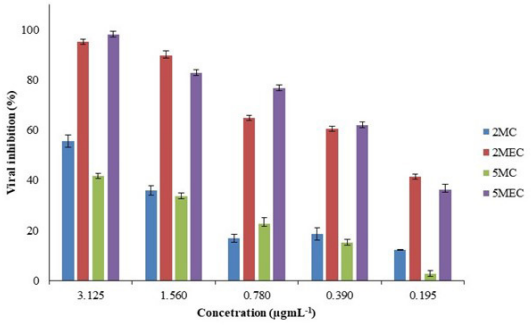

Figure 4. Results of the virucidal evaluation of the crude extract of *C. glabrata* microemulsioned against HSV-2 strain 333 by the plate reduction assay. 2 and 5MC: control microemulsion; 2 and 5 MEC: microemulsion with crude extract of *C. glabrata*. Results expressed on standard deviation of mean ± S.D.

compared to conventional systems such as gels, creams, and ointments (Callender et al., 2017; Poomanee et al., 2017).

During the stability analyzes, the physicochemical variations were evaluated by electrical conductivity, refractive index, pH, and viscosity. The same initial characteristics could be observed after the preliminary (14 days), in accelerated (90 days) stability tests, the results maintaining stable after the 60 days, showing conservation of the systems' profile until finish analyses.

The formulations kept at 40 ± 1° C showed a decrease in conductivity, since the increase in temperature favored the loss of water from the system, presumably due to the packaging material which was a glass vial with a rubber lid. No variations were observed in the formulations kept at 5 ± 1 °C (ANVISA, 2004). Therefore, the analyzes of the antioxidant, photoprotective, and virucidal potentials were conducted with the formulations maintained at a temperature of 25 ± 1 °C.

All formulations were classified as oil in water (O/W) systems because they have higher electrical conductivity values than distilled water (≥ 1.0 μScm^{-1}), showing that the incorporation of the extract did not affect the characteristics of microemulsified systems. This O/W orientation depends on the physicochemical characteristics of surfactants, such as their EHL. In order to have a microemulsion of the O/W type, it is necessary to use surfactants with EHL between 8 and 18, as it happens with TW, present in all MECs developed in this study (Rossi et al., 2007). No major changes in electrical conductivity were observed, demonstrating maintenance between the phases of the systems when compared with the MEC described by Ribeiro et al. (2015).

The pH values obtained for the formulations suggest stability, as variations in these values are indicative of instability, and are related to the presence of impurities, interaction with packaging materials, degradation and/or hydrolysis of the compounds present in the MEC (Torres et al., 2019; Subongkot and Sirirak, 2020).

Furthermore, the maintenance of the refractive index values during the stability tests (1.44 to 1.46) indicated the isotropic nature of all formulations. Changes such as an increase in the density of the system result in a slower

speed of light through the material, therefore, a higher refractive index. The opposite happens with a decrease in the density of the system (Parsi and Salabat, 2020).

The reograms (Figures 1 and 2) showed that the MECs presented Newtonian flow characteristics. Systems with this type of profile tend to have stability over a longer period of time (Ribeiro et al., 2015; Tagavifar et al., 2016).

Dynamic light scattering (DLS) is a useful technique to analyze the droplet diameter distribution of the formulations' internal phase (Callender et al., 2017). Formulation 5 MEC presented the best results with a smaller droplet diameter and lower PDI among the developed systems. The 4 MEC and 1 MEC showed smaller diameters, but the PDI values remained high, indicating less homogeneity in the droplet sizes of these systems. The 3 MEC showed a low PDI, however, the diameter was larger than the other formulations while the 2 MEC, when compared to the others, showed a small increase in diameter, but with a low PDI. The high concentration of surfactants helped to maintain stability, while the co-surfactant favored the dispersion of the extract and made it possible to form droplets with smaller sizes, which is one of the most important attributes for relating the particle size of a thermodynamically stable system (Ribeiro et al., 2016; Callender et al., 2017). The particles measurement obtained for the formulations was according to expected, considering that microemulsified systems have droplet size ranges smaller than 1000 nm (Figure 3) (Cunha Júnior et al., 2003).

The values obtained for the zeta potential for all formulations indicated the constancy of the systems, since the strong positive or negative charges present on the surface of the particles generate a repulsion force between them, ensuring that there is no coalescence between the formed droplets (Larsson et al., 2012; Callender et al., 2017; Ribeiro et al., 2020).

Within the genus *Cordia*, numerous biological activities in different types of extracts are reported, in addition to the existence of a herbal product from the species *C. verbenacea* (Al-Musayeib et al., 2011; Rahman and Akhtar, 2016). Thus, as pointed out by Debiasi et al. (2021) during phytochemical analysis, the ethanolic extract of *C. glabrata* showed the presence of phenolic compounds and flavonoids in its composition, as well as an antioxidant activity superior to other species from the same genus, and a potent virucidal activity (CV_{50} < 0.195 μgmL^{-1}) against HSV-2, making it interesting for incorporation into carrier systems since they can improve the extract's biological activities.

In the determination of phenolic acids by HPLC it was observed that the acids present in the extract maintained the concentration after being incorporated into the MEC. The determined phenolic compounds, collaborated with the antioxidant, antiviral and photoprotective activities of the extract (Nicácio et al., 2017).

The analysis of the antioxidant capacity by the DPPH radical scavenging method showed statistical difference between all formulations ($p < 0.05$), with the following decreasing sequence of antioxidant activity: 4 MEC> 2 MEC> 3 MEC> 5 MEC> 1 MEC. However, there was an improvement in the antioxidant capacity when compared to analyzes performed by Debiasi et al. (2021) in crude extracts of the *C. glabrata* leaves, which showed an EC_{50} of 49.79 ± 0.47 μgmL^{-1}. Concerning the $ABTS^{+}$ radical scavenging method, there was no statistical difference between 1, 2, 3, 4, and 5 MEC since these values remained close.

When compared to $ABTS^{+}$, the DPPH method stands out for being a rapid analysis and having greater precision and reproducibility when used to analyze plant extracts and isolated compounds. In addition to that, DPPH reacts emphatically with polyphenols while $ABTS^{+}$ reacts with a range of phenolic compounds (Mareček et al., 2017).

Several metabolites that perform biological activities (especially flavonoids) are involved in the photoprotection process of plants against UV radiation, arousing interest in the pharmaceutical industry for their incorporation into photoprotectors (Legouin et al., 2017; Oliveira-Júnior et al., 2017).

Brazilian legislation, through Collegiate Board Resolution (RDC) Nº 30, from June 1, 2012 (Brasil, 2012), establishes 6.0 as a minimum value for considering that a product has photoprotective activity. Thus, when confront the values previously obtained by Debiasi et al. (2021) in which they find a value of an SPF value of 5.41 ± 0.00 for the crude extract of C. glabrata, with the SPF values obtained after incorporating the extract into the MECs, there was a decrease in them presenting values between 2.14 to 2.84 ± 0.00. Although this decrease in the value of SPF, the MECs containing extract can still help in reducing the use of synthetic photoprotective compounds in cosmetic products, in order to minimize unwanted effects caused by them.

For the virucidal potential, two formulations (2 and 5 MEC) were chosen based on the general stability of the systems and better hydrodynamic particle sizes and PDI, since the amount of flavonoids were similar between the MECs. The SI values revealed good selectivity in the virucidal activity against HSV-2 for the analyzed MECs containing extract of *C. glabrata* (Figure 4).

The virucidal activity observed in the analyzed MECs containing the extract may be related to the denaturation of proteins responsible for the fixation of the HSV-2 virus in the host's cells, in addition to possible degradation of the virion structure (Álvarez et al., 2020).

Although a previous study published by the group (Debiasi et al., 2021) showed that the virucidal activity is higher for the crude extract, it should be taken into account that the MEC's studied contain a concentration of 1% of extract. Thus, the ability of the MECs containing extract to reduce HSV-2 infectivity in concentrations lower than its CC_{50}, as well as its best SI, suggests that these systems are promising for carrying the extract with the possibility of use in products for viral inactivation.

5. Conclusion

The developed microemulsions containing extracts of *C. glabrata* were stable in the face of quality control analysis and the rheological profile provided by the flow and viscosity curve, showing that all MECs presented Newtonian profile and demonstrated effectiveness in improving solubility and stability of the extract.

There was homogeneity in the size of the particles of the different MECs as well as significant zeta potential, which may prevent coalescence of the system phases due to presence of repulsion forces.

In the HPLC analyzes, the phenolic compounds quantified in the crude extract remained present in the same concentrations when incorporated into the microemulsions, which justifies the observed antioxidant activity. Although related to the antioxidant activity, the SPF results were below the minimum value required by ANVISA.

When submitted to the evaluation of virucidal activity, the MEC showed a better selectivity index against HSV-2 and good values of virucidal activity. Thus, the data revealed in this article are promising for the incorporation of extracts of *C. glabrata* for use in cosmetic and/or pharmaceutical products, due to the biological activities verified. On that matter, for the next stages of analysis, the flavonoid portion of the crude extracts will be isolated, identified, and evaluated concerning their biological activities.

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