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Original Article

Geopropolis gel for the adjuvant treatment of candidiasis – formulation and *in vitro* release assay

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ABSTRACT

The geopropolis produced by the stingless bee *Melipona subnitida* (popularly called "jandaíra" in Brazil) is a mixture of resin, wax, and mud. This study analyzed the antifungal activity of the geopropolis extract from *Candida* spp., developed a gel formulation with this extract and analyzed the delivery of bioactives (kinetics release) in the formulation and their chemical profile by UHPLC-PDA-qTOF-MS/MS. Three different gels were prepared using the geopropolis extract, carbomer, propylene glycol, and water. Formulations with different amounts of propylene glycol were investigated. Physical, visual, pH, viscosity, adhesion, spreadability, leakage, and *in vitro* release tests were performed in the proposed formulations. Antifungal tests with the geopropolis ethanolic extract were carried out against six *Candida* species. The chemical profile of the geopropolis extract and compounds released from the formulations were analyzed after the release test. The formulations had a pH between 4.6 and 4.8 and viscosity between 535,600 and 920,400 cPs. The geopropolis extract presented excellent antifungal activity against the tested yeasts. The results of the release test in semipermeable cellulose membrane showed that all formulations containing 5%, 10% and 40% propylene glycol presented release of geopropolis extract. For adhesion and leakage tests, the gel formulation with 5% propylene glycol was more effective. Both geopropolis ethanolic extract and the liquid obtained in the release test showed the presence of flavonoids (flavonol/flavone, flavanone, and chalcones). Gel formulations with geopropolis extract that are rich in flavonoids are promising as an adjuvant treatment of vaginal candidiasis.

Introduction

Melipona subnitida is a species of stingless bee popularly known as "jandaíra" in Brazil. It is a typical species of the northeastern Caatinga region and produces a variety of propolis known as geopropolis. This geopropolis is a mixture of resin, wax, and mud (Nogueira-Neto, 1997) and a product of meliponiculture with great pharmacological potential.

In recent years, the geopropolis produced by different species of stingless bees have shown therapeutic activities as anticancer (Bartolomeu et al., 2016), antioxidant (Alves De Souza et al., 2013; Dutra et al., 2014; Souza et al., 2014), anti-inflammatory (Franchin et al., 2012, 2013; Souza et al., 2014), gastroprotective (Ribeiro-Junior et al., 2015), antiviral (Coelho et al., 2015), and antimicrobial (da Cunha et al., 2013) agents. Our research group has studied the geopropolis of *M. subnitida*, and its chemical analysis showed phenolic compounds such as galloyl hexosides, ellagic acid, acyl-(cinnamoyl/coumaroyl)-hexosides, acyl-(cinnamoyl/coumaroyl)-galloyl-hexosides, and flavonoids (aglycones and acylated-*O*-glycosides) (Alves De Souza et al., 2013; de Souza et al., 2018). Two of these, (1,6-di-*O*-(*E*)-coumaroyl-2-*O*-galloyl-β-D-glucopyranoside and 1-*O*-cinnamoyl-6-*O*-(*E*)-coumaroyl-2-*O*-galloyl-β-D-glucopyranoside), were isolated as new compounds (de Souza et al., 2018).

Invasive fungal infections are a major concern for humans because they are associated with high mortality rates. Although the availability of antifungal drugs to combat invasive and superficial fungal infections has increased substantially over the past decade, they are not completely effective and generally present serious toxicity. Diseases caused by yeasts of the *Candida* genus have spread in recent times and with them, the concerns about treatment. Problems such as toxicity, limited spectrum, and the emergence of antifungal-resistant strains are common (Cameron et al., 1993; White et al., 1998; Mutlu Sariguzel et al., 2016).

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Candidiasis is a mycosis caused by yeasts of the *Candida* genus, which can cause mild or severe, acute or chronic, and superficial or deep lesions, however, all with quite variable clinical manifestation. The main candidiasis causative agent is the species *Candida albicans*, which constitutes about 60% of all isolates in clinical samples from hospital-acquired infection. The species *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. glabrata*, *C. kefyr*, *C. lusitaniae*, *C. viswanathii*, and *C. famata*, among others, have been isolated in clinical samples (Chaves et al., 2003). Candidal vulvovaginitis is caused by inflammatory changes in the vaginal and vulvar epithelium secondary to infection with *Candida* species, most commonly *C. albicans*. This process caused by the abnormal growth of yeasts in the female genital tract, becoming pathogenic (Barrenetxea, 2002).

The treatment of candidiasis includes the use of antifungal drugs, through topical and/or systemic use, and including three main chemical classes: polyenes (amphotericin B and nystatin), imidazoles (clotrimazole and miconazole), and triazoles (fluconazole and itraconazole) (Paiva et al., 2009). However, a high index of resistance to fluconazole and amphotericin B is observed, especially in patients who present recurrent infections. None of the antifungal agents currently available on the market represents the ideal drug for the treatment of candidiasis.

Along with ointments and creams, gels are semi-solid pharmaceutical forms intended for local administration and can be applied to the skin, on the eye surface, and even nasally, vaginally, or rectally. Gels are considered dispersions of small or large molecules in an aqueous liquid carrier, which acquires jelly-like consistency by the addition of a gelling agent. Among the gelling agents used are synthetic macromolecules such as carbomer, cellulose derivatives such as carboxymethylcellulose, and natural gums such as adragante gum (Allen et al., 2007). The main characteristic of a gel is its continuous structure, which confers properties similar to that of solids, where a natural or synthetic polymer builds a three-dimensional matrix through the hydrophilic liquid. Because this matrix presents an efficient function in carrying active substances such as pharmaceuticals or phytochemical complexes, and since some drugs do not bind to polymers, clay, or gums, it efficiently releases them at their sites of action. In addition, the formulation's pores allow the relatively free diffusion of smaller molecules.

The potential of geopropolis as an antifungal therapeutic alternative to current conventional therapies, along with the increasing spread of yeasts and fungal-resistant strains, prompted the objectives of the present study. Hence, this study analyzed the antifungal activity of the *M. subnitida* geopropolis extract against yeasts of the *Candida* genus in order to propose a gel-based pharmaceutical formulation for local administration, and evaluated the availability of bioactives (kinetics release) in the formulation and its chemical profile though UPLC-DAD-qTOF-MS/MS.

Materials and methods

General experimental procedure

The XEVO-G2XSQTOF mass spectrometer (Waters, Manchester, UK) was connected to the Acquity UPLC system (Waters, Milford, MA, USA) *via* an electrospray ionization interface (ESI). The analytical detector (Waters Acquity DAD detector) was set to a wavelength range of 200–400 nm. The chromatographic separation of compounds was performed on the ACQUITY UPLC with a conditioned autosampler at 4 °C using an Acquity BEH C18 column (50 mm × 2.1 mm i.d. and 1.7 μm of particle size) (Waters, Milford, MA, USA). All data acquisition and analysis were controlled using the Waters MassLynx v 4.1 software (Waters Corporation, Milford, MA, USA). To evaluation of the geopropolis gel formulations were used centrifuge (Centrifuges, Baby-I, 206 BL, FANEM, São Paulo, Brazil), pHmeter (PG 1800 Gehaka, São Paulo, Brasil), Viscometer (Model LVT, Brookfield Engineering Lab., Inc., Middleboro, MA), disintegration test apparatus (DIST-3, Pharma-Test, Hainburg, Germany), Tube-shaped dialysis (MWCO 12000–14000 – 5 M, Serva, Germany), dissolution tester basket (Dissolution tester rotating basket DT808-LH, Erweka, Germany), Spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer, São Paulo, Brazil), C-18 cartridges (1 g, SEP-PAK Waters). Thin layer chromatography was performed with pre-coated silica gel 60 PF254 plates from Merck (0.25 mm, Darmstadt, Germany). Formic acid, sodium hydroxide, propylene glycol, and Sabouraud dextrose was obtained from Merck (Darmstadt, Germany). Acetonitrile was obtained from Sigma (St Louis, MO, USA). Milli-Q water was used for UHPLC-DAD-qTOF-MS analysis. Ethanol (Tedia, Brazil) was of analytical grade. A Strata C18 (1 g) cartridge was employed to obtain phenolics (San Diego, CA, USA). Dimethyl sulfoxide (DMSO) was obtained from Vetec (Fine Chemistry), propylene glycol, Sabouraud dextrose broth, peptone water were purchased from Merck (Germany). All reagentes used were of analytical grade.

Samples and geopropolis extraction

The sample of *Melipona subnitida* geopropolis was collected in April of 2015 in the Riacho Farm, Vieirópolis city, in Paraíba State, Brazil. The geopropolis sample (527.8 g) was extracted with ethanol (98%) being drug:solvent ratio 1:3 in an ultrasound bath. The extract was filtered and concentrated using a rotary evaporator to provide the ethanolic extract (8.5 g). The geopropolis and ethanol extract were refrigerated at 4 °C until analyzed.

Analyses of the geopropolis by UHPLC-DAD-qTOF-MS/MS

The mobile phase consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) was pumped at a flow rate of 0.4 ml per min^{-1}. The gradient elution program was as follows: 0–5 min, 5–10% B, and 5–9 min, 10–95% B. The injection volume was 5 μl. The column temperature was maintained at 40 °C. The MS analysis was performed on a quadrupole time-of-flight tandem mass spectrometer coupled with an electrospray ionization source in the negative or positive ion mode. The scan range was from 50 to 1200 *m/z* for data acquisition. In addition, MS^E experiments were carried out, which allowed the acquisition of both precursor and product ion data in one injection. The source conditions were as follows: capillary voltage of 2.0 kV; sample cone source temperature of 100 °C; desolvation temperature of 250 °C; cone gas flow rate of 20 l/h; and desolvation gas (N_2) flow rate of 600 l/h. All analyses were performed using the Lock Spray, which ensured accuracy and reproducibility. Leucine-enkephalin (10 ng ml^{-1}) was used as a standard or reference compound to calibrate the mass spectrometers during analysis and introduced by a Lock Spray at 10 μl per min^{-1} for accurate mass acquisition.

Formulation of the gel containing geopropolis extract

Table 1 shows the ingredients used in the gel formulation with their compositions, proportions, and function. The gel base composition comprises carbomer, a high molecular weight synthetic polymer (Rowe et al., 2012) on an aqueous basis, plus preservatives, neutralizers, and co-solvents in which the geopropolis extract was incorporated. This formulation comprised a preliminary stage of pre-formulation studies when standardization was performed in the geopropolis extract followed by the selection of a polymer base, its concentration of use, preparation process, and pH correction. Three different gel formulations containing the same concentration of geopropolis extract were manipulated: carbomer gel formulation with 10% propylene glycol (F1), 40% propylene glycol (F2),

Table 1
Gel formulation with jandaíra geopropolis extract.

Formulation ingredients (% w/w)	Pharmacotechnical function[a]	F1	F2	F3
Carbomer 980	Polymer. Gelling agent, rheology modifier	1.5	1.5	1.5
Propylene glycol	Co-solvent	10	40	5
Methylparaben	Preservative, antimicrobial	0.18	0.18	0.18
Propylparaben	Preservative, antimicrobial	0.10	0.10	0.10
Geopropolis extract	Active substance	1	1	1
Water	Vehicle	sqf[b]	sqf[b]	sqf[b]

[a] Rowe et al. (2012).
[b] Sufficient quantity for 100%.

and 5% propylene glycol (F3). The release efficacy of these formulations was evaluated at different concentrations of co-solvent. The composition of the different formulations is summarized in Table 1.

The gel was prepared by mixing the carbomer 980 with water, stirring for 15 min. The preservatives methylparaben and propylparaben were dispersed with propylene glycol and water; the geopropolis extract was subsequently dispersed with propylene glycol and mixed in the formulation while stirring. The gel pH and viscosity were corrected with sodium hydroxide solution, added to make the final pH 4.5–5.0 (vaginal specific pH).

Evaluation of the geopropolis gel formulations

Physical examination and phase separation

The physical parameters visually analyzed in the formulations were appearance and color. The centrifugation assays of gel tubes were performed according to the Anvisa guidelines (Anvisa, 2007) to evaluate the quality and efficiency of the formulated gel mixture. Tubes containing gels were centrifuged at 700 × *g* for 40 min. Samples were subsequently visually analyzed for possible phase separation or precipitate formation.

pH determination

The pH of the formulated geopropolis gels was determined using a pHmeter calibrated by using standard buffer.

Viscosity measurement

Viscosity was determined in the formulations using the Viscometer at 25 °C with the spindle speed of 0.3 rpm.

Spreadability test

The spreadability of the gel of geopropolis was based on a method proposed by Borghetti and Knorst (2006). Briefly, 1 g of gel was placed within a circle of 1.2 cm diameter premarked on a glass plate over which a second glass plate was placed. A weight of 73 g was allowed to rest on the upper glass plate for 1 min. The increase in the diameter due to spreading of the gels was noted.

Adhesion test

The bioadhesive potential of geopropolis gel was evaluated in comparison to a commercially available vaginal gel (Kronel gel®). This test was according to (Bachhav and Patravale, 2009). A 50 mg of sample was placed at the center of an agar plate (1%, w/w agar in pH 4.5 citrate buffer). After 5 min, the agar plate was attached to an disintegration test apparatus and moved up and down in buffer 37 ± 1 °C. The plate remained immersed in the solution during the whole test. The residence time of gels on the plate (adhesion time) was visually determined.

Leakage test

The test was performed according to Ochoa Andrade et al. (2014). Geopropolis gel (80 mg) (diluted in physiological solution pH 4.5) were syringed onto one end of an agar glass slide (1%, w/w agar). The agar slide was attached to one of the inner walls of a transparent chamber, which was maintained at 37 °C in a water bath. The slide remained in the vertical position at an angle of 90° to the horizontal for 2 h. The running distance of the gel along the slide was measured against squared paper.

In vitrorelease studies of the geopropolis gel formulations

The developed formulations were submitted to *in vitro* release kinetics assays to assess the release profile of bioactive compounds (Balata et al., 2014). The test was performed with an artificial cellulose acetate membrane for tube-shaped dialysis. The membranes were previously immersed in the recipient medium (same medium used on the receiving side), consisting of phosphate buffered saline, pH 4.5–5.0 (vaginal specific pH) and methanol (9:1), for a period of 24 h. Two grams of each gel formulation were applied uniformly into the membrane (over an area measuring about 9 cm^2), the membrane was closed and coupled to the dissolution tester basket so that the outer surface of the membrane remained with greater contact surface with the receptor medium. The submerged membranes were subjected to a fixed speed of 50 rpm. The recipient compartment consisted of 500 ml of medium maintained at 37 + 0.5 °C throughout the experiment. Aliquots (10 ml) were taken at 10, 30, and 45 min, and at every hour at regular intervals for a period of 8 h. These aliquots were analyzed by spectrophotometry at 270 nm for chemical composition. Both negative (receptor medium without sample) and positive (receptor medium with the geopropolis ethanolic extract) controls were used. The dissolution medium was replaced after each collection.

The analysis of chemical constituents released into the receptor medium was also performed by UPLC-DAD-qTOF-MS/MS; the result was compared with the geopropolis ethanolic extract before the gel formulation. The liquid was subjected to solid phase extraction in C-18 cartridges to recover the chemical constituents of the receptor medium at the end of the experiment; these cartridges were previously conditioned with 10 ml of methanol and 10 ml of deionized water. The liquid resulting from the gel experiment was passed through the cartridge, rinsed with 10 ml of water, and compounds were eluted with 10 ml of HPLC-grade methanol. The eluate was dried under reduced pressure in a rotatory evaporator at 40 °C, dissolved in methanol, filtered through a 0.45-μm Nylon syringe filter, and injected into the UPLC-DAD-qTOF-MS/MS system.

The following analyses were performed to investigate the release kinetics of bioactive compounds in the geopropolis gel: cumulative percentage of extract release *versus* time (zero-order kinetic model), log of the cumulative percentage of extract release *versus* time (first-order kinetic model), and cumulative percentage of extract release *versus* the square root of time (Higuchi model). Data were analyzed statistically by the linear regression method,

and the release kinetics was defined by the graph that presented the best linear correlation coefficient (r) close to 1 (Costa and Sousa Lobo, 2001).

Antifungal activity of the geopropolis extract against Candida yeasts

The antifungal activity was determined in six species of the *Candida* genus that are detected in vaginal candidiasis. The ethanolic extract of geopropolis was tested in the American Type Culture Collection (ATCC) strains of the following microorganisms: *C. albicans* (ATCC 18804), *C. krusei* (ATCC 34135), *C. glabrata* (ATCC 2001), *C. tropicalis* (ATCC 13803), *C. guilliermondii* (ATCC 6260), and *C. parapsilosis* (ATCC 22019). The yeast strains were inoculated in solid medium Nutrient Agar (Merck) and maintained at 32.5 ± 2.5 °C in a bacteriological oven for 48 h as pre-cultures. The standardization of the inoculum (CLSI, 2009) consisted in the preparation of a yeast suspension in 0.1% sterile peptone (Merck) with turbidity of 0.5 in the McFarland scale, corresponding to approximately 1×10^6 CFU/ml (for yeasts). The fungal suspension was diluted in 0.1% peptone water at 1:100, followed by another 1:20 dilution of the standard suspension, yielding a suspension of 2.5×10^3 CFU/ml, which was used in the assays.

The geopropolis ethanolic extracts were prepared with 10 mg/ml DMSO and subsequently diluted in the appropriate concentrations for the assays; the final concentration of DMSO was 10%. The Minimal Inhibitory Concentration (MIC) was determined by the plate microdilution technique using U-shape 96 wells plates and according to the methodology described by the CLSI, standard M27-A2.

A total of 80 μl of Sabouraud dextrose broth was added to each well. A total of 100 μl of the testing solutions (geopropolis extracts) were added in the first row of wells; successive dilutions were carried out down the rows to determine the Minimum Inhibitory Concentration (MIC). Finally, 20 μl of the microorganism suspension was added to all wells. Plates were covered with lids, wrapped with plastic film, and incubated at 32.5 ± 2.5 °C for 24 h in a bacteriological oven. The following concentrations of geopropolis extract were tested diluted in Sabouraud broth in the 96 wells plates: 500; 250; 125; 62.5; 31.25; 15.62, and 7.8 μl/ml. Samples were developed with resazurin sodium (25 μl 0.01% in water), and plates were incubated for 2 h at 32.5 ± 2.5 °C before readings and analysis. Ketoconazole was used as the positive control; solutions without compound or extract were used as the negative control. The MIC was defined as the lowest concentration of the antimicrobial agent capable of preventing visible microorganism growth in the microdilution plate assay.

Statistical analysis

All experiments were performed in triplicate. All values were expressed as average ± standard deviation. All statistical analyses were performed using the software package (GraphPad InStat Demo Version). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Before the gel formulations development, the geopropolis ethanolic extract was submitted to antifungal tests to evaluate its capacity to inhibit the growth of yeasts in the *Candida* genus, which is responsible for vaginal candidiasis. The extract's chemical profile was obtained by UPLC-DAD-qTOF-MS/MS concomitantly to the antifungal tests. The sample showed activity, and the *C. albicans* and *C. tropicalis* strains were the most sensitive to the geopropolis extract (Table 2).

Table 2
In vitro activity of the geopropolis ethanolic extract of the jandaíra bee (*Melipona subnitida* Ducke) against strains of Candida.

Samples	Minimum inhibitory concentration (MIC) Minimum fungicide concentration (MFC) mg/ml											
	Candida albicans ATCC 18804		*Candida krusei* ATCC 34135		*Candida glabrata* ATCC 2001		*Candida tropicalis* ATCC 13803		*Candida guillermondii* ATCC 6260		*Candida parapsilosis* ATCC 22019	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Geopropolis extract	250	1.000	500	1.000	500	1.000	250	1.000	500	1.000	500	1.000
Cetoconazol	0.12	31.25	0.24	62.5	0.24	62.5	0.12	31.25	0.24	62.5	0.24	62.5

Table 3
Evaluation of gel formulations containing jandaira geopropolis extract.

Formulation	Aspect	Color	Phase separation	pH	Viscosity (cp)
F1	1	Greenish brown	Nil	4.80 ± 0.14	748.800 ± 1.002
F2	1	Greenish brown	Nil	4.60 ± 0.09	535.600 ± 1.090
F3	1	Greenish brown	Nil	4.70 ± 0.23	920.400 ± 1.032

1: Viscous gel, homogeneous, with the characteristic odor of geopropolis extract.

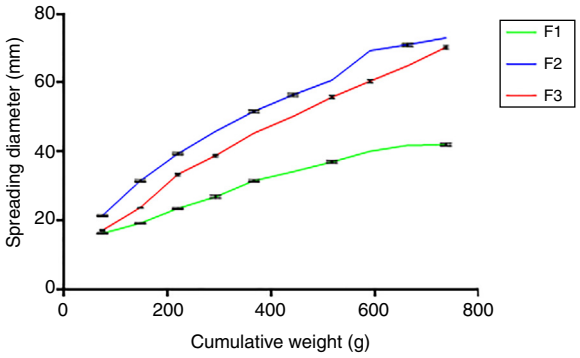

Fig. 1. Spreadability of gel formulations containing jandaira geopropolis extract.

Although there are several species of native bees producing geopropolis in Brazil, few chemical and biological studies have been carried out. The geopropolis of *Melipona fasciculata* has been demonstrated to inhibit the growth of *C. albicans* (Liberio et al., 2011). The geopropolis ethanolic extract produced by *Melipona quadrifasciata anthidioides* from the Central Western region of Brazil presented activity against yeast strains including the *C. albicans* strain, which is resistant to hospital drugs (Dos Santos et al., 2017). Geopropolis is a product of meliponiculture with great pharmacological potential. These activities depend on the geopropolis chemical composition, which in turn depends on the local flora, type of bee, and the type of soil (Alves De Souza et al., 2013; de Souza et al., 2018).

Among the pharmaceutical forms used in the local treatment of conditions and pathologies in the vulvovaginal area are ointments, creams, foams, and gels. The vagina surface is lined with squamous epithelial cells and mucus produced by several glands. Local vaginal products are used to treat infections, vaginitis, conditions of endometrial atrophy, and contraception by the use of spermicides. These products come into direct contact with tissues that are prone to infections; therefore, these products must be free of pathogens such as bacteria and fungi, and should be packed in specific tubes and packages, and applied to the vagina through specific tips (Allen et al., 2007).

Carbomers may be used in formulations as emulsifiers, suspenders, solid agglutinating agents, and viscosity modifiers. They can be dispersed in water to form acidic colloidal solutions of low viscosity and viscous gels when neutralized (Corrêa and Júnior, 2005). At the concentrations of 0.5–2% in water, carbomers are used as gelling agents added to pharmaceuticals, solvents such as propylene glycol, antimicrobials, and stabilizers, without interference in their rheological properties (Allen et al., 2007). Carbomer 980 was chosen in this study as the gel base for the incorporation of the geopropolis extract because it presents excellent viscosity parameters, compatibility with several active complexes, bio-adhesive properties, thermal stability, good patient acceptability, and excellent organoleptic characteristics (Goodrich, 1997; Islam et al., 2004). The acidic nature of carbopol causes the formulation pH to decrease, resulting in reduced ionization of carboxylic groups and increased winding of polymer molecules. This fact reflects a marked reduction in viscosity, gel strength, and mucoadhesion (Morsi et al., 2017); therefore, the formulations tested in the present study required pH correction and consequently viscosity correction. In the specific case of products for vaginal application, the pH must be according to the application site in order to prevent its interference in normal vagina physiological processes or in the unbalance of inherent microbiota (Allen et al., 2007). The higher the viscosity of formulations for local administration, the better the bio-adhesion mechanism at the application site when compared to less viscous formulations. The final viscosity in the gel formulation proposed in the present study was specified as a consequence of the amount of neutralizing agent applied for pH correction, *i.e.*, decrease in pH along with the viscosity. The resulting viscosity seemed ideal when compared to other proposed formulations (Chorilli et al., 2007), promoting good spreadability and robustness in the phase separation tests. Substances or bioactive complexes with apolar characteristics, incorporated into gels, will have a greater affinity for the receptor fluid (mucosae) than for the polymer base, contributing to an improved release. Thus, during the development of formulations incorporated into gels, their affinity for the base must be considered to predict the release profile of an active agent from a carrier (Chorilli et al., 2007). El-Menshawe et al. (El-Menshawe et al., 2017) studied the development of a nanostructured gel formulation, noting that increasing propyl-

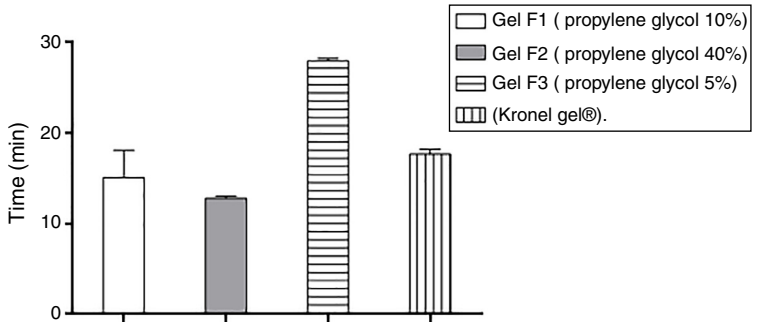

Fig. 2. Adhesion time of gel formulations containing jandaira geopropolis extract in pH 4.5 citrate buffer.

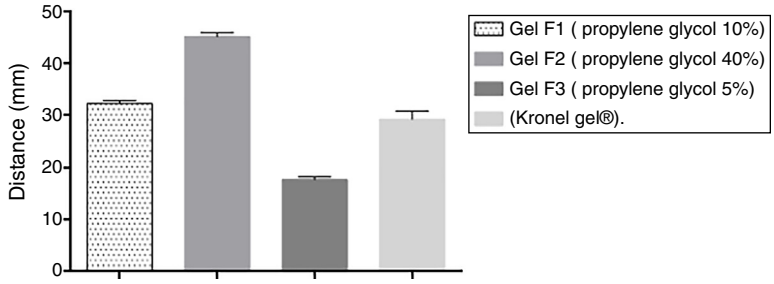

Fig. 3. Leakage distance of gel formulations containing jandaira geopropolis extract.

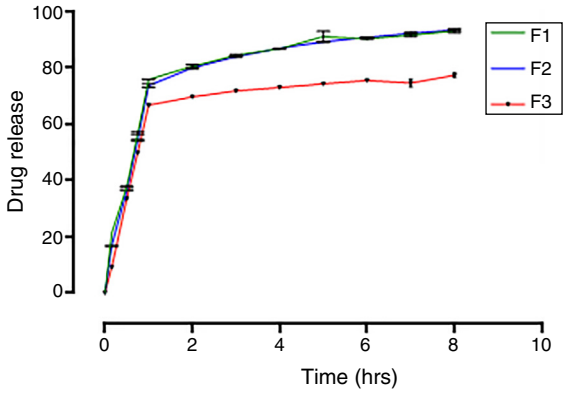

Fig. 4. *In vitro* release assays of gel formulations containing jandaira geopropolis extract through cellulose membrane in phosphate buffered saline pH 5.

ene glycol by 20% in these formulations slows the flow of drug release.

The physical-chemical parameters presented by the formulated gels are presented in Table 3. The formulation shows a viscous gel appearance; it is homogeneous, with a greenish-brown color, and has the characteristic geopropolis odor. All formulations remained homogeneous in the phase separation test without precipitate formation, phase separation, sedimentation, or coalescence. The pH initially presented values around 3.0 and were corrected to 4.8 (F1), 4.6 (F2), and 4.7 (F3) by the addition of sodium hydroxide, yet remaining within the pH range ideal for vaginal use (4.5 and 5.0) and not impacting the homeostasis of the vaginal microbiota (Allen et al., 2007). Viscosities were between 535,000 cPs and 920,400 cPs. Spreadability is an important property of local formulation to assure uniform application, dosage transfer and therapeutic efficacy. The diameters found in the tests to formulations are indicative of good spreadability, being the formulations with 10% and 40% of propylene glycol better (Fig. 1). In the adhesion and leakage tests it was observed that the lower the amount of propylene glycol in the geopropolis gel the better the adhesion (Fig. 2) and the lower leakage (Fig. 3). It's important to accomplish the retention of a formulation on a mucous membrane and potential to assure a prolonged action, respectively.

The *in vitro* release studies were performed using a cellulose membrane. The cumulative percentage of geopropolis extract release by the different formulations over an 8-h period is shown in Fig. 4. It is observed that the maximum release of the geopropolis extract from the formulations occurs within 8 h. The three gel formulations show a release level between 66% and 75% in the first

Table 4
Kinetic data from the release assays of gel formulations containing jandaira geopropolis extract.

	Zero-order		First-order		Diffusion model (Higuchi)	
Formulation	R^2	K_0	R^2	K_1	R^2	K_h
F1	0.9619	11.59	0.7784	0.56	0.9619	33.2
F2	0.9635	11.67	0.8060	0.56	0.9634	33.2
F3	0.9300	9.63	0.8060	0.54	0.9285	27.5

hour, thus demonstrating an important burst effect as the extract remains free in the gel. An increased release of geopropolis extract in the gel was observed in the formulation with 10% propylene glycol (F1); the formulation containing 40% propylene glycol (F2) presented an F1-like release profile. The decrease of 5% propylene glycol (F3) in the gel reduced the geopropolis extract release, being this decrease of the release of the extract of the geopropolis statistically not significant. These kinetic values are shown in Table 4. According to the results, all formulations were linear, with regression coefficients ranging from 0.9635 to 0.9619.

The chemical profile of the geopropolis ethanolic extract was determined by UPLC-DAD at 340 nm (Fig. 6); the compounds tentatively identified by UHPLC-QTOF-MS/MS (Fig. 5) were flavonoids (flavonol/flavone, flavanone, and chalcones) based on their characteristic UV–vis spectra peaks and mass detection as well as accurate mass measurements of the precursor and product ions. All flavonoids detected are listed in Table 5. Twenty-six flavonoids were identified, of which nineteen flavones/flavonols (2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 19, 20, 22, 23, 24, and 25), five flavanones (1, 5, 15, 16, and 21), and two chalcones (14 and 26). Flavonoids 3, 5, 9, and 21 were compared to authentic standards. Several groups of flavonoid isomers such as compounds 3, 15 (301 Da), 2, 7, 14, and 21 (285 Da), 8, 10, and 18 (315 Da), 6, 16, 26 (269 Da), 12, 13, 19 (329 Da), 8, 10 (299 Da), 17, 23, 24 (313 Da), and 20, 25 (343 Da) were present in the geopropolis.

The test to verify the release of compounds by the formulation and passage through the cellulose membrane showed that all flavonoids present in the geopropolis extract were present in the liquid after release by the membrane (Fig. 6), which shows that the geopropolis is rich in flavonoids and may be used in formulations for the adjuvant treatment of vaginal candidiasis. The anti-candida activity can be attributed to the blending of various flavonoids present in the geopropolis. The flavonoid classes identified in this study are already known to have antifungal activity against *C. albicans* (Seleem et al., 2017), including flavonoids isolated from the propolis collected by *Apis mellifera* (Herrera et al., 2010; Agüero et al., 2014) (Figs. 5 and 6).

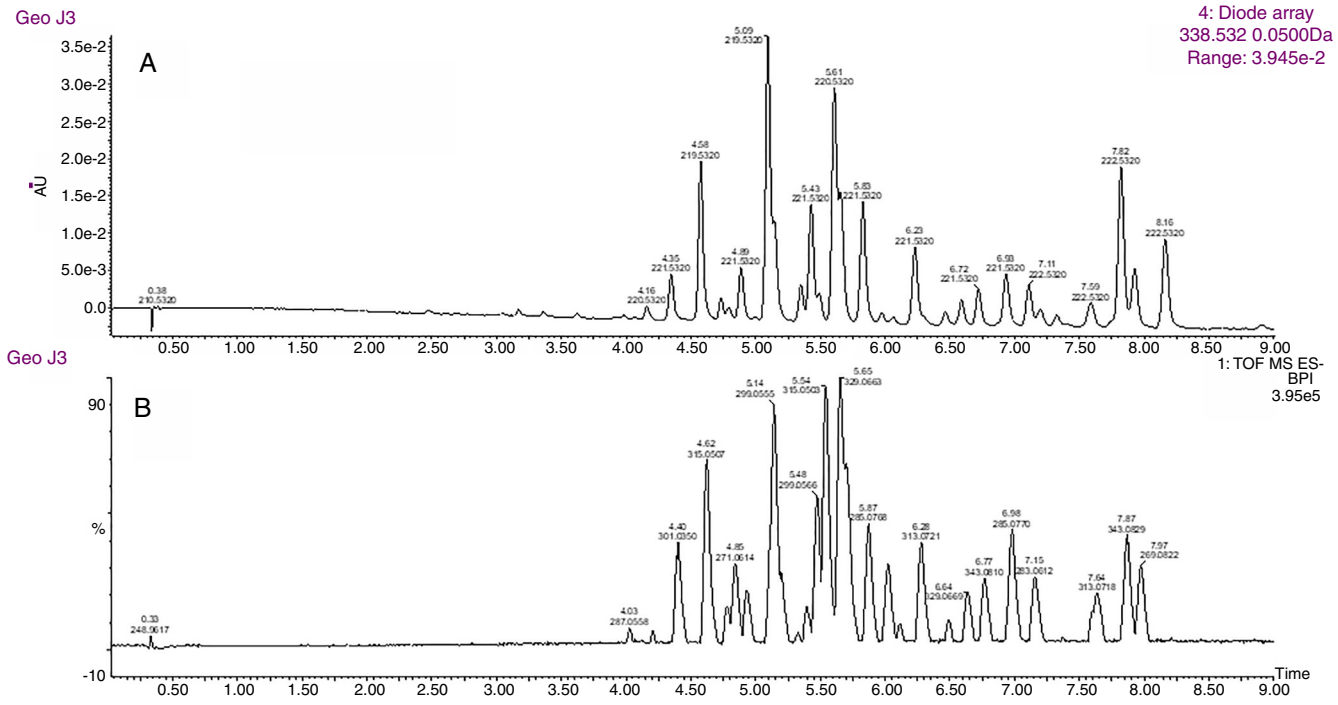

Fig. 5. UPLC-DAD chromatogram (340 nm) of *Melipona subnitida* geopropolis extract (A). Base peak ion (BPI) chromatogram analyzed by UPLC-QTOF-MS, ESI in mode negative (B).

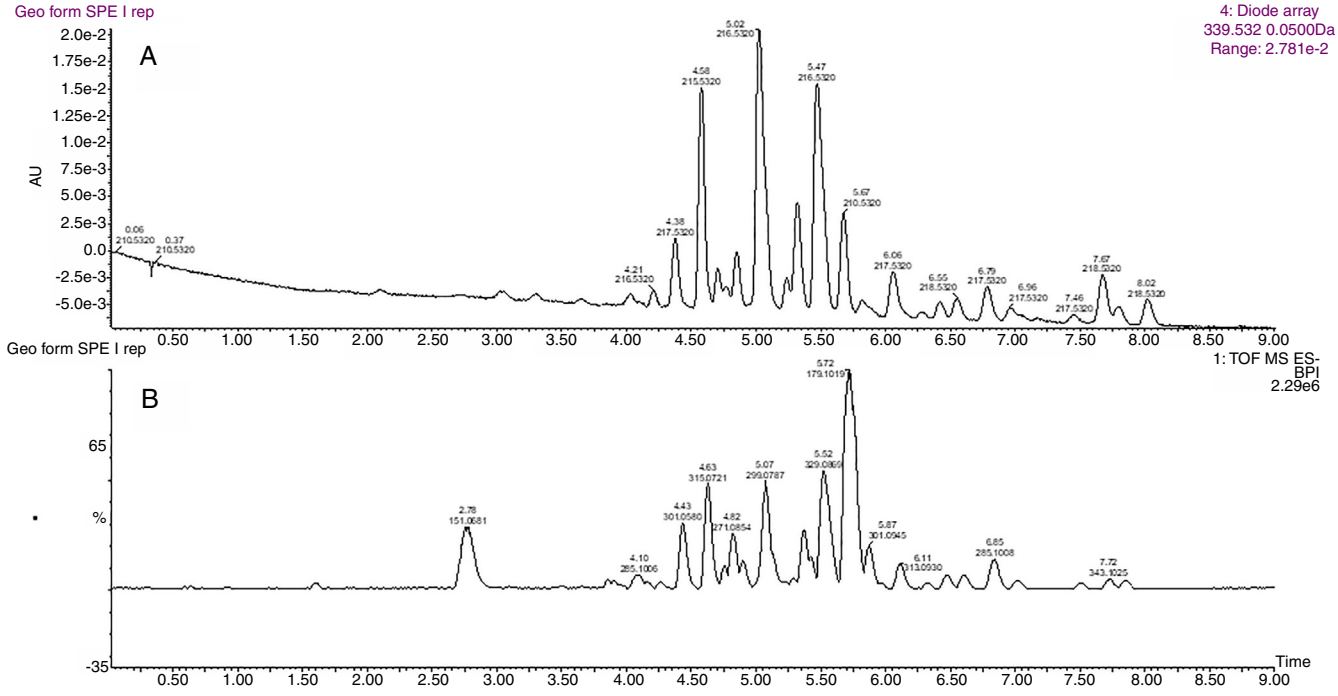

Fig. 6. UPLC-DAD chromatogram (340 nm) of liquid after release by the membrane with *Melipona subnitida* geopropolis extract formulation (A). Base peak ion (BPI) chromatogram analyzed by UPLC-QTOF-MS, ESI in mode negative (B).

Table 5
Characterization of compounds from *Melipona subnitida* geopropolis ethanolic extract by UPLC-DAD-QTOF-MS^E.

	RT (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	$[M-H]^-$ (m/z) Calculated	Tentative identification
1	4.03	287	285.0761	287.0761	Tetrahydroxy-flavanone
2	4.20	345	285.0404	285.0404	Tetrahydroxy-flavone
3[a]	4.40	356	315.0512	315.0510	3-*O*-methyl-quercetin[a]
4	4.77	345	345.0615	345.0615	Myricetin dimethyl-ether
5[a]	4.85	287	271.0610	271.0611	Naringenin[a]
6	4.93	339	269.0455	269.0455	Trihydroxy-flavone
7	5.08	364	285.0405	285.0404	Tetrahydroxy-flavone
8	5.48	346	299.0563	299.0561	Trihydroxy-methoxy-flavone
9[a]	5.15	346	315.0504	315.0510	Isorhamnetin[a]
10	5.22	346	299.0563	299.0561	Trihydroxy-methoxy-flavone
11	5.54	287	315.0515	315.0510	Tetrahydroxy-methoxy-flavone
12	5.65	345	329.0662	329.0667	Quercetin dimethyl ether
13	5.70	345	329.0660	329.0667	Quercetin dimethyl ether
14	5.87	369	285.0768	285.0768	Hydroxy-methoxy-chalcone
15	6.01	286	301.0718	301.0717	Trihydroxy-methoxy-flavanone
16	6.11	275	269.0826	269.0819	Hydroxy-methoxy-flavanone
17	6.28	333	313.0716	313.0716	Dihydroxy-dimethoxy-flavone
18	6.48	356	315.0513	315.0510	Tetrahydroxy-methoxy-flavone
19	6.64	360	329.0666	329.0667	Pentahydroxy-flavone
20	6.77	339	343.0822	343.0823	Dihydroxy-trimethoxy-flavone
21[a]	6.98	287	285.0765	285.0768	7-*O*-methyl naringenin (sakuranetin)[a]
22	7.15	339	283.0618	283.0612	Dihydroxy methoxy flavone
23	7.37	346	313.0721	313.0718	Dihydroxy-dimethoxy-flavone
23	7.64	345	313.0718	313.0718	Dihydroxy-dimethoxy-flavone
25	7.87	339	343.0829	343.0823	Dihydroxy-trimethoxy-flavone
26	7.97	363	269.0822	269.0819	Dihydroxy-methoxy-chalcone

[a] Compared with the standard sample.

Conclusion

The local administration gel formulation of jandaira geopropolis extract is an effective formulation, which is rich in bioactive flavonoids, presents high antifungal activity, and can be used as an alternative therapeutic to the adjuvant treatment of candidiasis.

Authors' contributions

UPSJ (PhD student) contributed in biological study and analysis of the dates biological. CAC and TMC designed the chemical study, supervised the laboratory work, identification of compounds chemical and contributed to critical reading of the manuscript. EMS contributed in collecting samples propolis and identification. TMGS and SPC contributed in chemical study.

Conflicts of interest

The authors declare no conflicts of interest.

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