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Chemical composition, antinociceptive and free radical-scavenging activities of geopropolis from *Melipona subnitida* Ducke (Hymenoptera: Apidae: Meliponini)

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Abstract

Like many stingless bee species, *Melipona subnitida* Ducke uses geopropolis (a mixture of wax, plant resins, pollen grains and mud) for sealing small crevices in their nest cavities, in order to avoid the entry of air, and for defense against pathogenic microorganisms. The aim of this study was to evaluate the antinociceptive and free radical-scavenging activities of ethanolic extracts of six geopropolis samples from *M. subnitida* and the phenolic fractions obtained by C18-SPE extraction. The *in vivo* antinociceptive activity was analyzed on abdominal constriction induced by acetic acid in mice and *in vitro* free radical-scavenging activities by DPPH and ABTS assays. Additionally we analyzed the chemical composition of the phenolic fractions by HPLC-DAD. The six samples of geopropolis showed variations in the total phenolic content over the period, but not in the chemical profile observed by HPLC-DAD. Geopropolis is a rich source of bioactive compounds as phenolics 6-*O*-*p*-coumaroyl-*D*-galactopyranose, 6-*O*-cinnamoyl-1-*O*-*p*-coumaroyl-β-*D*-glucopyranose, 7-*O*-methyl naringenin, 7-*O*-methyl aromadendrin, 7,4'-di-*O*-methyl aromadendrin, 4'-*O*-methyl kaempferol, 3-*O*-methyl quercetin, 5-*O*-methyl aromadendrin and 5-*O*-methyl kaempferol with potential antioxidant and antinociceptive activities. The antioxidant activity is related to the total phenolic content.

Introduction

Many stingless bee species (Meliponini) store in their nests a large amount of geopropolis, a mixture of wax, plant resins, pollen grains and mud (Nogueira-Neto, 1997). The bees use this material for sealing small crevices in their nest cavities, in order to avoid the entry of air, and for defense against pathogenic microorganisms (Simone-Finstrom & Spivak, 2010). However, despite its popular use in folk medicine, very little is known about its chemical composition and biological activity.

Recently, studies investigating geopropolis from native bees have indicated a potential for bioactive compounds and biological activities. Velikova et al. (2000) analyzed 21 samples of Brazilian geopropolis from 12 different species of stingless bees and observed the presence of compounds such as di- and triterpenes and gallic acid. The same samples showed activity against *Staphylococcus aureus* Rosenbach and cytotoxic activity. Samples of *Melipona fasciculata* Smith geopropolis showed activity against *Streptococcus mutans* Clarke (Liberio et al., 2011) and antioxidant capacity (Dutra et al., 2014) and eleven compounds were tentatively identified as belonging to the classes of phenolic acids and hydrolysable tannins (gallotannins and ellagitannins). These compounds were responsible for the antioxidant activity and high phenolic content of the geopropolis produced by *M. fasciculata* (Dutra et al., 2014). Geopropolis produced by *Melipona scutellaris* Latreille has been shown to exhibit antimicrobial and antioxidant activities and has anti-inflammatory, antinociceptive and antiproliferative properties (Franchin et al., 2012; Cunha et al., 2013), and benzophenones have been identified as the major compounds (Cunha et al., 2013).

Previous investigations in our laboratory have found that the geopropolis from *Melipona subnitida* Ducke has antioxidant activity. This study led to the isolation and characterization of two phenylpropanoids, one of which was a new compound, and

seven flavonoids (Souza et al., 2013). These findings suggested that *M. subnitida* geopropolis is highly bioactive and deserved further study to identify other potential biological activities. Thus, the aim of this study was to evaluate the antinociceptive and free radical-scavenging activities of ethanolic extracts of six geopropolis samples from *M. subnitida* and its phenolic fractions. Additionally, we analyzed the chemical composition of the phenolic fractions obtained by C18-SPE extraction by HPLC-DAD.

Materials and methods

Geopropolis samples and fractionation

For this study, six samples of geopropolis from four *M. subnitida* nests were collected in March 2010 (**1**), July 2011 (**2**), January 2012 (**3**), April 2012 (**4**), June 2012 (**5**) and July 2012 (**6**) at Sítio Riacho Vieirópolis (a semi-arid region), Paraíba State, Brazil. Each sample (200 g) was extracted with 100 mL of ethanol (EtOH) in an ultrasonic water bath. The combined ethanolic extracts were completely evaporated under reduced pressure to a brown residue (2.7 g to 18.4 g). The EtOH extract (100 mg) was dissolved in 2 mL of distilled water, and the solution was adjusted to pH 2.0 by adding concentrated HCl while stirring with a magnetic stirrer at room temperature for 10 min. A C18 cartridge (SPE Strata 1 g, Phenomenex) was sequentially conditioned with 3 mL of MeOH and 6 mL of distilled deionized water without allowing the cartridge to dry. The samples of geopropolis were passed through the cartridge and rinsed with 6 mL of water and the phenolic compounds were eluted with 8 mL of HPLC-grade methanol. The eluate was dried under reduced pressure in a rotatory evaporator at 40 °C to yield 32 to 57 mg of phenolic fraction. These fractions were dissolved in methanol, filtered through a 0.45-µm nylon syringe filter (Whatman) and injected into the HPLC system. The phenolic samples were reconstituted with Tween® 80 and carboxycellulose and also to evaluation for their antinociceptive and antioxidant activities.

Reagents and standards

All reagents used were of analytical grade. Folin-Ciocalteu's phenol reagent, DPPH (1,1-diphenyl-2-picryl hydrazyl), potassium persulfate and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were supplied by Acros Organics (Belgium). ABTS (2,2 azinobis 3-ethylbenzothiazoline-6-sulfonic acid) was purchased from Fluka Chemie GmbH (Switzerland). Ascorbic acid was from Vetec (Brazil). Formic acid (Merck) and methanol (Tedia) were of analytical grade. Dipirone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), gallic acid, carboxymethylcellulose-CMC (Sigma); tween® 80 (Sigma-Aldrich, USA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). The compounds 6-*O*-*p*-coumaroyl-*D*-galactopyranose (**1**), 6-*O*-cinnamoyl-1-*O*-*p*-coumaroyl-β-*D*-glucopyranose (**2**), 7-*O*-methyl naringenin (**3**), 7-*O*-methyl aromadendrin (**4**), 7,4'-di-*O*-methyl aromadendrin (**5**), 4'-*O*-methyl kaempferol (**6**), 3-*O*-methyl quercetin (**7**), 5-*O*-methyl aromadendrin (**8**) and 5-*O*-methyl kaempferol (**9**) had been previously isolated and identified from *M. subnitida* geopropolis (Souza et al., 2013).

HPLC analysis of the phenolic

All chromatographic analyses were performed using a Shimadzu Prominence LC-20AT equipped with a SPD-M20A diode array detector (Shimadzu Corp. Kyoto, Japan). The samples (20 µL) were injected into a Rheodyne 7125i injector with a 20 µL loop. The column heater was set at 40 °C. The chromatographic separation was performed with a Luna Phenomenex C-18 column (250 mm x 4.6 mm x 5 µm). The compounds were separated using a mobile phase consisting of 1% aqueous formic acid (A) and methanol (B) at a flow rate of 1 mL/min. The mobile phase was delivered using the following solvent gradient: 0-10 min, 20-25% B; 10-20 min, 25-60% B; 20-30 min, 60-70% B; 30-35 min, 70-100% B. The injection volume was 20 µL. Chromatograms were recorded at 290 nm and 340 nm. The identification of the compounds was based on their retention times and UV spectra with authentic markers.

Animals

Male and female Swiss mice weighing 20-25 g were used and given access to water and food *ad libitum*. We used six mice per experimental group. The animals were housed at a temperature of 25-28°C with a 12 h light/12 h dark cycle. The procedures described were reviewed and approved by the local Animal Ethics Committee (CEUA UFAL process number 23065.004873/2011-01).

Determination of the total phenolic content

The total phenolic content of the samples was determined with the Folin Ciocalteu reagent, according to the method of Slinkard and Singleton (1977), modified by using gallic acid as a standard phenolic compound. EtOH extracts (100 µL) and phenolic fractions (1 mg/ml) were transferred to an Eppendorf tube with 1 ml. Folin Ciocalteu reagent (20 µL), 820 µL of distilled water were added and the contents of the flask were mixed thoroughly. After 1 min, 60 µL of sodium carbonate (15%) was added and then the mixture was allowed to stand for 2 h. The absorbance was measured at 760 nm with an automatic Biochrom Asys UVM 340 microplate reader (Cambridge, UK). The amount of total phenolic compounds was determined in micrograms of gallic acid equivalents using the equation obtained from the standard gallic acid graph.

DPPH• radical scavenging assay

The free radical-scavenging activity was determined using the DPPH assay, as described previously (Silva et al., 2006) with

modifications. The antiradical activity was evaluated using a dilution series to obtain five concentrations (1.0 to 80.0 µg/mL). This process involved mixing the DPPH solution (23.6 µg/mL in EtOH) with the appropriate EtOH extracts and phenolic fractions followed by homogenization. After 30 min, the remaining DPPH radicals were quantified by measuring the absorption at 517 nm with an automatic Biochrom Asys UVM 340 microplate reader (Cambridge, UK).The percentage of inhibition was given by the formula: percent inhibition (%) = [(A0 - A1)/A0] x 100, where A0 was the absorbance of the control solution and A1 was the absorbance in the presence of the sample and standards.

$ABTS^{\bullet+}$ radical cation decolorization assay

The radical cation decolorization assay was based on the method described by Re et al. (1999) with modifications. ABTS was dissolved in water to yield a final concentration of 7 mM. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting the ABTS stock solution with 2.45 mM of potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 0.70 ± 0.05 at 734 nm with ethanol before use. Then, appropriate amounts of the $ABTS^{\bullet+}$ solution were added into 0.5 mL of the sample solutions in ethanol at five concentrations (1-40 µg/mL). After 10 min, the percentage inhibition of the absorbance at 734 nm was calculated for each concentration with an automatic Biochrom Asys UVM 340 microplate reader (Cambridge, UK), relative to the blank absorbance (EtOH). The capability to scavenge the $ABTS^{\bullet+}$ radical was calculated using the following equation: $ABTS^{\bullet+}$ scavenging effect (%) = [(A0-A1/A0) x100], where A0 was the initial concentration of the $ABTS^{\bullet+}$ and A1 was absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of sample.

Evaluation of activity ethanol extracts and fractions of geopropolis on abdominal constriction responses caused by acetic acid

Abdominal constrictions (writhes) were induced by the i.p. injection of acetic acid (1.2%) and carried out according to the procedure described previously (Koster et al., 1959; Collier et al., 1968; Fontenele et al., 1996). Mice were treated with EtOH extracts and phenolic fractions (100 mg/kg, i.p.) or Dypirone (10 mg/kg, i.p.) 40 minutes before initiating nociceptive stimulus. Dypirone was used as a positive control and the vehicle (CMC/Tween® 80) (10 mL/kg, i.p.) was used as the negative control (the animals without treatment). The total numbers of writhes, which consisted of constriction of the flank muscles associated with inward movements of the hind limb or with whole body stretching, were counted cumulatively over a 20 min period. The antinociceptive activity was determined as the difference in number of writhes between the control group and the treated group.

Statistical analysis

All analyses were performed in triplicate. The results were expressed as the standard error of the mean (mean ± S.E.M.) and were analyzed using GraphPad Prism 5.0 program (DEMO). Comparisons between groups were made using analyses of variance (ANOVA) followed by Tukey's test. Significance was indicated by a p value ≤ 0.05. Pearson's correlation test was used to evaluate the correlations.

Results and Discussion

The aim of this study was to evaluate the antinociceptive activity of six samples of *M. subnitida* geopropolis collected over three years. EtOH extracts and the phenolic fractions were evaluated in a model of nociception, and the free radical-scavenging activity was evaluated using the DPPH and ABTS assays. The total phenolic content was determined by the Folin Ciocalteu reagent. In addition, chromatographic profiles were analyzed by HPLC-DAD, and the principal phenolics present in the geopropolis samples were identified.

This study was conducted by an extraction of phenolics using a C18-SPE cartridge as a simpler, less expensive and faster technique compared with the use of liquid-liquid solvent extraction. This technique has been used to determine flavonoid markers in honey (Hadjmohammadi et al., 2009). Interestingly, there is a correlation ($r=0.85$, $p<0.05$) between the total phenolic content present in the ethanolic extract and the amount of phenolics extracted by C18-SPE. These samples showed a total phenolic content two times higher when compared with the EtOAc fraction (which is rich in phenolic compounds) obtained by the liquid-liquid extraction of a sample of *M. scutellaris* geopropolis collected in January 2010 (Souza et al., 2013). The phenolic profiles of samples **1-6** also were analyzed by HPLC-DAD. The characterization of these compounds is important because they are associated with a variety of health benefits. The comparative analysis of the chromatograms (Fig 1) shows a similar profile between the six samples obtained by the SPE and the EtOAc fraction (Souza et al., 2013) of geopropolis, again demonstrating that SPE extraction is effective for extraction of phenolics. All phenols (phenylpropanoids and flavonoids) previously identified from EtOAc fraction (Souza et al., 2013) were verified in the samples of this study; the 6-*O*-*p*-coumaroyl-*D*-galactopyranose compounds (**1**), 6-*O*-cinnamoyl-1-*O*-*p*-coumaroyl-β-*D*-glucopyranose (**2**), 7-*O*-methyl naringenin (**3**), 7-*O*-methyl aromadendrin (**4**), 7,4'-di-*O*-methyl aromadendrin (**5**), 4'-*O*-methyl kaempferol (**6**), 3-*O*-methyl quercetin (**7**), 5-*O*-methyl aromadendrin (**8**) and 5-*O*-methyl kaempferol (**9**) were identified (Fig 1).

Further studies are necessary to quantify the compounds identified. The following plant species occur in the region and are resin-producing sources possibly collected by the bees for propolis production: *Myracrodruon urundeuva* Allemão (Anacardiaceae), *Handroanthus impetiginosus* (Mart. & DC.) Mattos (Bignoniaceae), *Jatropha mollissima* (Pohl) Baill.

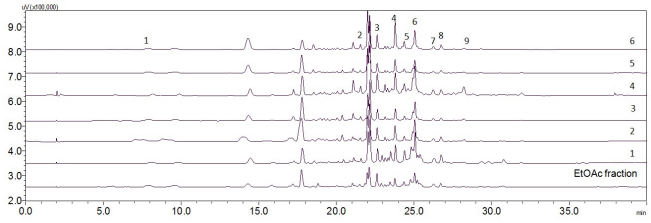

Fig 1. Chromatograms (HPLC-DAD 320 nm) of the phenolic fractions of *Melipona subnitida* geopropolis (1-6) and of EtOAc fraction geopropolis collected in January 2010. Compounds identified were: 6-*O*-*p*-coumaroyl-*D*-galactopyranose (**1**), 6-*O*-cinnamoyl-1-*O*-*p*-coumaroyl-β-*D*-glucopyranose (**2**), 7-*O*-methyl naringenin (**3**), 7-*O*-methyl aromadendrin (**4**), 7,4'-di-*O*-methyl aromadendrin (**5**), 4'-*O*-methyl kaempferol (**6**), 3-*O*-methyl quercetin (**7**), 5-*O*-methyl aromadendrin (**8**) and 5-*O*-methyl kaempferol (**9**)

(Euphorbiaceae) and *Anadenanthera colubrina* (Vell.) Brenan (Fabaceae) (Maia-Silva et al., 2012). Other studies to verify the presence of pollen in *M. subnitida* geopropolis are required, because pollen analysis in addition to chemical analysis is a method used to characterize regionally different propolis samples. Pollen types that occur in low frequency in propolis samples can be regarded as an indicator of the botanical species supplying the resin (Matos et al., 2014). It is a good tool for defining the phytogeographical origin of resins and quality of the propolis (Barth et al., 2003). Barth et al. (1999) and Barth and Luz (2003) showed that there is a fairly equal number of pollen grains between the samples of propolis from *Apis* and geopropolis produced by Meliponini, but a wider richness of pollen types is characteristic of geopropolis. In this regard, the Meliponini visits more plant species than the *Apis* bees. Nevertheless, the occurrence of dominant and accessory pollen grains is more frequent in propolis samples, which reflects a higher generalization of honeybees.

Evaluating abdominal constrictions induced by acetic acid was initially used to evaluate the antinociceptive activity of the EtOH extracts (100 mg/kg) of geopropolis and their phenolics fractions (100 mg/kg). The results showed in Fig. 2A and Table 1 demonstrate that the EtOH extract (100 mg/kg), produced inhibition of abdominal constrictions induced by acetic acid in mice ($p<0.05$), with inhibitions of 96.9% (sample 5) to 100% (sample 1). Phenolic fractions at the same concentration also inhibited the number of writhes ($p<0.05$) from 71.4% (sample 3) to 93.5% (sample 5), Fig 2B and Table 1. The inhibitory properties of the EtOH extracts and the phenolic fractions versus the abdominal constrictions induced by acetic acid in mice is first suggestion of the antinociceptive potential of these materials. The acetic acid induced constrictions test is a typical model for inflammatory pain that has long been used as a screening tool for the assessment of analgesic properties. The fact that the EtOH extracts showed slightly greater antinociceptive activities than the phenolic fractions suggests that geopropolis contains other compounds responsible for this activity and should be chemically investigated. The phenolic fraction is probably principally responsible for this activity. No reports on antinociceptive activity have been found in the literature for the identified constituents of *M. subnitida* geopropolis.

Table 1. Effects of injections of ethanolic extracts and phenolic fractions of geopropolis on abdominal constrictions induced by acetic acid in mice.

Samples	Numbers of writhers			
	EtOH extracts		Phenolic fractions	
	Media ± S.E.M.[a]	% inhibition[b]	Media ± S.E.M.[a]	% de inhibition
Control	38.4 ± 2.7	-		
Dipirone	18.8 ± 2.7	29.9 *		
1	0.0 ± 0.0	100.0 ***	4.5 ± 1.0	85.4 ***
2	0.2 ± 0.2	99.4 ***	8.2 ± 1.6	73.5 ***
3	0.2 ± 0.2	99.4 ***	8.8 ± 2.5	71.4 ***
4	0.7 ± 0.3	97.5 ***	3.0 ± 1.9	90.3 ***
5	0.8 ± 0.6	96.9 ***	2.0 ± 0.7	93.5 ***
6	0.2 ± 0.2	99.4 ***	5.2 ± 3.1	83.2 ***

[a] Data are expressed as the mean ± SEM, n=6. [b] Symbols indicate significant difference ((*P<0.05 and ***P<0.001, One Way ANOVA followed by Dunnett's test) compared to control group. Control was treated with vehicle (CMC/Tween® 80) (10 ml/kg, i.p.), dypirone 100 mg/kg, i.p. 40 minutes before initiating nociceptive stimulus.

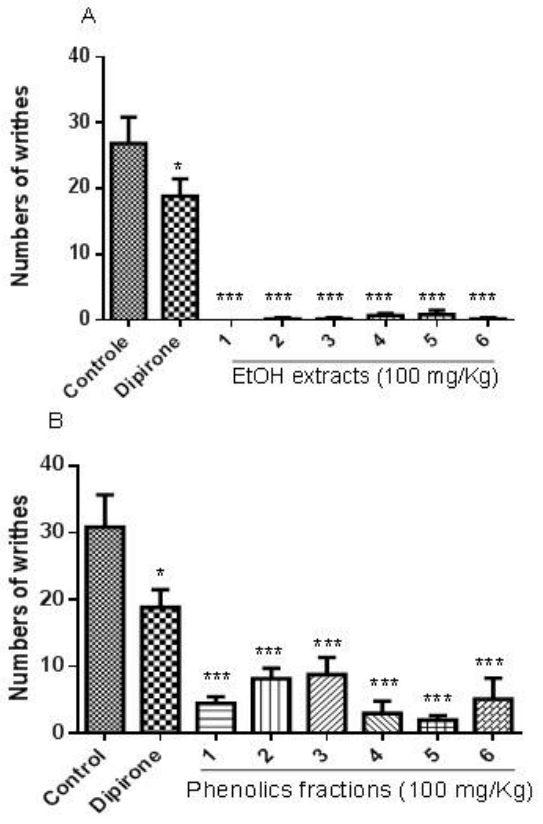

Fig 2. Effects of injections of ethanolic extract of geopropolis and phenolic fractions on abdominal constriction induced by acetic acid in mice. Control groups included the mice treated with only vehicle (negative control) or dypirone (positive control) 40 min before initiating nociceptive stimulus. Data are expressed as the mean ± SEM, n=6. Symbols indicate significant differences (*P<0.05 and ***P<0.001, One Way ANOVA followed by Dunnett's test) compared to the control group.

The free radical-scavenging activities of the EtOH extracts and phenolic fractions from geopropolis are shown in Table 2. The CE_{50} ranged from to 6.99-15.2 µg/mL (ABTS) and 13.3-39.2 µg/mL (DPPH) for the EtOH extracts and 3.2-8.9 µg/mL (ABTS) and 7.5-17.1 µg/mL (DPPH) for the phenolic fractions. The lower EC_{50} value indicates a higher antioxidant activity. The EtOH extracts and phenolic fractions showed a correlation between free radical-scavenging activity and the total phenolic content. The phenolic content ranged from 92.6-201.6 to EtOH extract and 205.5 to 305.3 to phenolic fractions. A correlation between DPPH-ABTS results for the EtOH extracts (r=0.91) and the DPPH-ABTS results for the phenolic fraction (r=0.97) was observed (Table 3).

These results suggest that total phenols, particularly the phenylpropanoids and flavonoids identified in *M. subnitida* geopropolis were responsible for the free radical-scavenging activity. Geopropolis obtained from the other stingless bees showed important antioxidant activities (Silva et al, 2013; Dutra et al, 2014). In early studies other *M. subnitida* products such as the pollen (Silva et al, 2006) and honey showed (Silva et al, 2013) free radical-scavenging activity. The pollen collected by the stingless bees *Melipona rufiventris* Lepeletier (Silva et al, 2009) and honey produced by *Melipona seminigra merrillae* Cockerell (Almeida da Silva et al., 2013) also were reported as having important antioxidant activities.

Table 2. Total phenolic and free radical-scavenging activity of *M. subnitida* geopropolis samples.

Geo-propolis sample	Total phenolic content (mg GAE/g ± SD)		ABTS[a] CE_{50} (µg/mL)		DPPH[a] CE_{50} (µg/mL)	
	EtOH extract	Phenolic fraction	EtOH extract	Phenolic fraction	EtOH extract	Phenolic fraction
1	97.6 ± 5.7	273.9 ± 6.8	15.2± 0.8	4.3 ± 0.1	39.2 ± 0.9	8.4 ± 0.1
2	92.6 ± 8.1	204.5 ± 7.4	13.4 ± 0.7	8.9 ± 0.7	31.7 ± 0.5	17.7 ± 0.2
3	172.6 ± 4.5	305.3 ± 5.0	7.7 ± 0.1	3.4 ± 0.1	15.9 ± 0.4	7.6 ± 0.1
4	150.7 ± 5.1	282.4 ± 1.5	10.3 ± 0.2	4.5 ± 0.2	16.1 ± 0.4	9.8 ± 0.1
5	201.6 ± 4.2	322.4 ± 6.4	6.9 ± 0.3	3.1 ± 0.1	13.3 ± 0.4	7.5 ± 0.1
6	139.3 ± 6.9	261.3 ± 5.8	15.2 ± 0.5	6.0± 0.1	28.9 ± 1.2	10.5 ± 0.1
Ascorbic acid			-		2.8 ± 0.0	2.8 ± 0.4
Trolox			3.21 ± 0.0	3.21 ± 0.0	-	-

[a] Mean value ± standard deviation: n=3, Concentration of antioxidant required to reduce the original amount of the radicals by 50%.

Table 3. Pearson correlation coefficients between the total phenolic content and the antiradical activity DPPH and ABTS.

	DPPH		ABTS	
	EtOH extracts	Phenolic fractions	EtOH extracts	Phenolic fractions
Total Phenolic ContentEtOH extracts	-0.90		-0.85	
DPPHEtOH extracts			0.91	
ABTSEtOH extracts	0.91			
Total Phenolic Content phenolic fractions		-0.94		-0.97
DPPH phenolic fractions				0.97
ABTS phenolic fractions		0.97		

Conclusion

The present results from six samples of *M. subnitida* geopropolis collected over three years showed that there is a variation in the total phenolic content over the years but not in the chemical profile. Geopropolis is a rich source of bioactive compounds with potential antioxidant and antinociceptive activities. The antioxidant activity is related to the total phenolic content. The SPE extraction was effective for the extraction of phenolic from *M. subnitida* geopropolis.

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