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Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: Use of correlation and multivariate analyses to identify potential bioactive compounds☆

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ABSTRACT

The production of propolis by honeybees results from a selective collection of exudates from various plant species and present many potentialities in the pharmaceutical industry. The objective of this study was to investigate the chemical profile of Brazilian propolis, as well as their in vitro antioxidant and antibacterial activities. Gas chromatography–mass spectrometry was applied for chemical profiling of propolis extracts. Total phenolic compounds were quantified by Folin-Ciocalteu and antioxidant properties were assessed by 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. Antibacterial activity was assessed against *Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus luteus*. Correlation and multivariate statistical analysis were used to identify potential bioactive compounds in the extracts. Twenty-nine metabolites were identified along with 34 other metabolites that were classified into the following classes: triterpenoids (12), acetyltriterpenoids (3), sesquiterpenes (6), steroids (4), and hydrocarbons (9). The antioxidant capacity (IC_{50}) ranged from 21.50 to 78.77 μg/mL, whereas the content of total phenolic compounds ranged from 31.88 to 204.30 mg GAE/g of dry weight. Total phenolic compounds and methyl retinoate showed a positive correlation with the antioxidant capacity, whereas tetradecanal, γ-palmitolactone and ethyl hydrocinnamate showed a negative correlation. Different sets of metabolites are shown to correlate with the antibacterial activity of the extracts, which is largely dependent on the type of microorganism. This innovative approach allowed us to identify likely bioactive compounds in the extracts, although the mechanism(s) underlying antibacterial activity encompass a complex trait, which might involve synergistic effects.

1. Introduction

Propolis is an apicultural term for the resins harvested from various plant species by honey bees when used within a hive (Simone-Finstrom & Spivak, 2010) and it is considered a promising source of compounds for the discovery of new pharmaceuticals. In the last decades, several works dealing with propolis' composition and biological properties have been published, revealing the interest of researchers on this product and its potential for the pharmaceutical industry (Alves & Kubota, 2013; Bankova, De Castro, & Marcucci, 2000; Castro et al., 2009; Costa Da Silva, Muniz, De Cássia Saraiva Nunomura, Nunomura, & Zilse, 2013; Gonçalves, Santos, & Srebernich, 2011). Its chemical composition is tightly associated with the ecology of the region and plant species from which the nectar is collected, as well as with the genetic variability of the queen bee (Cheng, Qin, Guo, Hu, & Wu, 2013; Daugsch, Moraes, Fort, & Park, 2008; Kumazawa, Hamasaka, & Nakayama, 2004; Kumazawa et al., 2003; Park, Alencar, & Aguiar, 2002; Park, Paredes-Guzman, Aguiar, Alencar, & Fujiwara, 2004; Santos et al., 2003). Daugsch et al. (2008) investigated the chemical profile of Brazilian red propolis in order to determine their botanical origin. They observed that bees were collecting the reddish exudates on the surface of *Dalbergia ecastophyllum*. The red propolis contained many flavonoids, such as formononetin, rutin, liquiritigenin, daidzein,

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and quercetin, which were also present in the resinous exudates from the surface of *D. ecastophyllum*. In another study, the botanical origin of Brazilian propolis was investigated using reversed-phase high-performance thin-layer chromatography (RPHPTLC), reversed-phase high-performance liquid chromatography (RPHPLC), and gas chromatography–mass spectrometry (GCMS) (Park et al., 2002). Several compounds were identified, including hydroxycinnamic acid derivatives (coumaric acid and ferulic acid), flavonoids (pinobanksin, isosakuranetin, and kaempferol), monoterpenes (α- and β-pinene), and some fatty acids (hexadecanoic acid, eicosanoic acid, 10-octadecenoic acid, and 9,12-octadecadienoic acid). Based on their chemical profile, it was concluded that the propolis were produced from the resins of *Hyptis divaricata* and *Baccharis dracunculifolia* (Park et al., 2002).

The green propolis is one the most popular type of propolis in Brazil. Its green color is derived from the chlorophyll collected by the bees which is present in young tissues and non-expanded leaves of *B. dracunculifolia* (Guimarães et al., 2012; Lemos et al., 2007). The green propolis is rich in flavonoids (e.g. ramnocitrin, eupalitin, and acacetin) and terpenoids (e.g. α- and β-amyrin, lupeol) (Tavares, Gomes de Lemos, Campos Arriaga, Pinheiro Santiago, & Braz-Filho, 2010). The brown propolis (also called propolis type 6) is mainly produced in the Northeastern Brazil from *H. divaricata* (Park et al., 2002). This propolis is characterized by the absence of flavonoids, presence of fatty acids such as oleate, palmitate, linoleate, and steareate, as well as by its remarkable inhibitory effect on bacterial growth (Castro et al., 2009).

Many biological properties have been attributed to propolis, including anticancer (Mouse et al., 2012), antimicrobial (Popova, Silici, Kaftanoglu, & Bankova, 2005), antioxidant (Alves & Kubota, 2013), antileishmanial (Duran, Muz, Culha, Duran, & Ozer, 2011), wound healing (Batista et al., 2012; Guney, Karaman, Oner, & Yerer, 2011), anti-inflammatory and immunomodulatory activities (Araujo, Libério, Guerra, Ribeiro, & Nascimento, 2011). For example, Mouse et al. (2012) investigated the chemical composition of Moroccan propolis extracts by HPLC/ESI-MS, as well as their in vitro and in vivo anticancer potential. It was shown that Moroccan propolis extracts contained several flavonoids, including rhamnetin, luteolin, and trihydroximethoxiflavone, some flavonoid glucosides, such as wogonoside, quercetin arabino-glucoside, and kaempferol O-glucoside and that the anticancer activity depended mainly on not only the chemical composition of the extracts, but also on the target tumor cells (Mouse et al., 2012). Using a GC–MS based approach, Duran et al. (2011) investigated the relationship between the chemical composition extracts obtained from two Turkish propolis and their antileishmanial activity. Several compounds were identified including some fatty acid esters (ethyl oleate), cinnamic acid esters (cinnamyl cinnamate), hydrocarbons (heptadecane, 1-heptadecane, and 1-nonadecene), and a sesquiterpene (δ-cadinene). These compounds contributed to the efficacy of the Turkish propolis extracts against *Leishmania infantum* and *Leishmania tropica* (Duran et al., 2011).

Studies based on bioassay-guided chemical analysis represent a very promising trend in propolis research and its antimicrobial activities are well documented against different bacteria (Braca, Siciliano, D'Arrigo, & Germanò, 2008; Cabral, Oldoni, de Alencar, Rosalen, & Ikegaki, 2012; Castro et al., 2009; Fabri, Nogueira, Dutra, Bouzada, & Scio, 2011; Gonçalves et al., 2011). For example, Popova et al. (2005) investigated the chemical composition of Turkish propolis extracts by GC–MS and reported that major chemical constituents of Turkish propolis were flavonoids (pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin, galangin) and hydroxycinnamic acid derivatives (p-coumarin acid, ferulic acid, benzyl p-coumarate, benzyl ferulate, and phenylethyl caffeate). The antibacterial activity of the propolis extracts showed good correlation with the concentration of phenolics, flavones and flavanones (Popova et al., 2005). Very few studies, however, used correlation and multivariate statistical analysis to identify potential bioactive compounds in the extracts. Thus, studies that correlate propolis biological activities, chemical composition and therapeutic action are of pivotal importance.

In the last decades, the interest in the antioxidant properties of plant-derived products, such as honey and propolis, has increased, mainly due to the known implications of reactive oxygen species (ROS) in oxidative stress and damage of cell components (Dizdaroglu & Jaruga, 2012; Du & Gebicki, 2002). Cellular protection against the deleterious effects of ROS can be achieved by means of antioxidant enzymes or by low molecular weight antioxidants such as vitamins C and E, carotenoids, reduced glutathione and phenolic compounds (Pereira, Ribeiro, Loureiro, de Castro, & Fernandez, 2014; Tewari, Singh, & Watanabe, 2013). The use of powerful chromatographic tools are essential for the analysis of complex mixtures, such as propolis, enabling the identification and quantification of their biologically active constituents (Tavares et al., 2010), however, there are few studies that investigated the chemical composition of propolis coupling with correlation and multivariate analyses to identify potential bioactive compounds. Thus, in this paper we describe the successful use of gas chromatography–mass spectrometry (GC–MS) for chemical profiling and characterization of propolis collected in different regions of Brazil, as well as their in vitro antioxidant and antibacterial activities. Correlation and multivariate statistical analysis were used to identify potential bioactive compounds.

2. Materials and methods

2.1. Sample preparation

Twenty five grams of each propolis were extracted (three consecutive times) by maceration with 100 mL of 95% ethanol at room temperature (Supplementary Fig. 1). After maceration was performed, the ethanol was removed under reduced pressure at 40 °C yielding 15.23 g of the fraction green propolis in ethanol (GPE) and 12.75 g of the fraction brown propolis in ethanol (BPE). Five grams of either GPE and BPE were dissolved in a mixture of ethanol and water (8:2) and partitioned with hexane to produce the sub-fractions green propolis in hexane (GPH, 1.46 g) and brown propolis in hexane (BPH, 1.79 g). Subsequently, partitioning of the remaining GPE and BPE solution with dichloromethane was performed to produce green propolis in dichloromethane (GPD, 1.62 g) and brown propolis in dichloromethane (BPD, 1.31 g).

2.2. Extraction of secondary metabolites for GC–MS analysis

We used the gas chromatography–mass spectrometry (GC–MS) for the analysis because this technique is very sensitive, simple and fast. Secondary metabolite profiling was performed with an Agilent 7809A gas chromatograph (Agilent Technologies) coupled to a Triple-Axis detector (Agilent 5975C), using a ZB-5 (Phenomenex; 30 m × 0.25 mm) capillary column (0.25 mm film thickness) using helium as a carrier gas at a flow rate of 1 mL/min as described by Ribeiro, Fernandez, de Castro, Ligterink, and Hilhorst (2014). Approximately 5 mg of the extracts were transferred to a 12-mL tube and 5 mL of a methanol:chloroform (1:1) solution was added. The resulting solution (100 μL) was transferred to a 2-mL glass GC vial with a 200-μL glass insert. Dodecane was used as internal standard. All analyses were measured using three biological replicates.

2.3. GC–MS Data processing and compound identification

Data processing and compound identification were performed as described by Ribeiro et al. (2014) and Ribeiro et al. (2015). Raw data were processed by ChromaTOF software 2.0 (Leco Instruments) followed by alignment between chromatograms using the MetAlign software (Lommen, 2009). MSClust was used to remove metabolite signal

redundancy in aligned mass peak tables and to retrieve mass spectral information of metabolites, using mass peak clustering (Tikunov, Laptenok, Hall, Bovy, & de Vos, 2012). The mass spectra of the representative masses were used for tentative identification by matching to the spectral libraries (National Institute of Standards and Technology [NIST08]; Golm metabolome database [http://gmd.mpimp-golm.mpg.de/]) and by comparison of the retention index calculated using a series of alkanes. Authentic reference standards were used to confirm the identity of the metabolites. Levels of identification are presented in Table 1 and Supplemental Table 1 as described by Sumner et al. (2007).

2.4. DPPH radical scavenging assay

The antioxidant properties were assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by Costa, Ribeiro, Castro, and Fernandez (2013) and Pereira et al. (2014). Initially, 1 mL of DPPH (120 mM; methanol) was added to 1 mL of each extract (20, 40, 80 and 160 μg/mL; methanol) to provide 2 mL of the reaction mixture with a final concentration of 10 to 80 μg/mL of the extract. After 30 min the absorbance values were measured at 517 nm and represented as IC_{50}. Methanol was used as a blank and DPPH solution (1.0 mL; 120 mM) plus methanol (1 mL) was used as negative control.

2.5. Total phenolic content

Total phenolic content was assessed as described by Pereira et al. (2014) with minor modifications. Initially, 100 μL of the extract (1 mg/mL; methanol) was mixed with 500 μL of Folin–Ciocalteu's phenol reagent and 6 mL of water. After 1 min, 1 mL of sodium carbonate solution (15% w/v) was added to the mixture, which was adjusted to 10 mL with distilled water. The reaction mixture was kept in the dark for 120 min and then the absorbance was read at 725 nm (Analyser 850M). Gallic acid standard curve was used to calculate total phenolic content in the extracts and the results are expressed as mg GAE/g of dry extract.

2.6. Antibacterial activity

The determination of minimum inhibitory concentration (MIC) of the extracts was performed by using the successive micro-dilution assay in 96-well plates as described by Ribeiro, Ferraz, Guedes, Martins, and Cruz (2011). Nutrient broth was used as culture media and chloramphenicol was used as positive control. The tested concentration of the extracts ranged from 500 μg/mL to 3.90 μg/mL and from 100 μg/mL to 0.78 μg/mL for the control. Samples were incubated for 24 h at 36 °C and the MIC was determined through the emergence of turbidity in the wells. Antibacterial activities of the extracts were assessed against *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633) and *Micrococcus luteus* (ATCC 10240). All analyses were performed in triplicate. Extracts were considered active when growth inhibition at concentrations below or equal to 500 μg/mL was observed.

2.7. Univariate and multivariate statistical analysis

Normalized data were uploaded at MetaboAnalyst 2.0, a web-based analytical pipeline for high-throughput metabolomics studies (http://www.metaboanalyst.ca/MetaboAnalyst/) (Xia & Wishart, 2011). Before data analysis, a data integrity check was performed to make sure that all the necessary information was collected. Row-wise normalization was performed to allow general-purpose adjustment for differences among samples. Log transformation and auto-scaling were performed to make features more comparable. Uni- and multivariate analysis were performed using log transformed and auto-scaled data.

Table 1
Relative metabolite concentration based on the normalized data of the metabolite profile of green and brown Brazilian propolis samples.

		Brown propolis			Green propolis		
Metabolite	RI	BPE	BPD	BPH	GPE	GPD	GPH
δ-Cadinene (1)	1525	1378.7 ± 21.4	ND	2650.1 ± 2.2	8646.3 ± 176.5	956.0 ± 27.1	19385.5 ± 175.9
Caryophyllene (1)	1431	3584.9 ± 77.8	ND	6754.6 ± 78.8	1399.0 ± 22.3	ND	6947.7 ± 185.0
Caryophyllene oxide (1)	1630	ND	ND	ND	10317.4 ± 500.3	9072.9 ± 227.5	15779.9 ± 198.2
Cis-oleic acid (1)	2146	5094.2 ± 191.6	642.9 ± 16.6	20230.8 ± 1590.8	3325.6 ± 57.3	976.1 ± 16.5	17899.5 ± 221.5
Cycloartenol (2)	4024	9334.5 ± 65.7	3923.3 ± 23.4	22615.6 ± 174.4	3676.3 ± 65.2	ND	6609.5 ± 101.4
Dihydrobenzofuran (1)	1213	ND	ND	ND	89563.0 ± 4797.9	119101.1 ± 3453.2	5947.2 ± 340.0
Epimanool (2)	2095	55425.2 ± 1490.5	7229.6 ± 147.9	113757.9 ± 1686.2	2610.2 ± 219.0	1126.0 ± 3.7	14638.3 ± 379.1
Ethyl hexadecanoate (1)	1992	6312.7 ± 210.0	339.5 ± 14.3	2592.3 ± 85.9	35759.3 ± 530.3	59844.4 ± 780.8	5504.4 ± 72.8
Ethyl hydrocinnamate (1)	1341	2091.2 ± 44.3	1930.2 ± 67.8	2179.9 ± 11.0	800231. ± 8172.6	139396.4 ± 370.1	1264626.1 ± 5587.4
Ethyl oleate (2)	2176	47131.6 ± 1865.8	1987. ± 21.4	23894.3 ± 290.0	19375.2 ± 500.6	3163.5 ± 532.8	21754.5 ± 424.6
Ethyl tetracosanoate (2)	2781	19727.7 ± 1713.4	481.1 ± 45.0	16936.9 ± 421.7	22115.1 ± 434.8	ND	29808.7 ± 686.0
γ-Muurolene (2)	1482	855.3 ± 22.6	ND	1837.2 ± 18.4	3972.9 ± 10.9	ND	17879.1 ± 229.0
γ-Palmitolactone (2)	2121	10358. ± 338.5	439.0 ± 27.1	23310.0 ± 337.5	14719.6 ± 384.5	3954.4 ± 42.8	83218.2 ± 705.0
Hexadecanal (2)	1822	674.4 ± 36.9	548.0 ± 39.7	547.4 ± 31.9	1916.1 ± 62.3	756.9 ± 10.4	13918.1 ± 203.0
Hexadecanoic acid (1)	1963	10416.2 ± 1156.9	2290.1 ± 247.8	47790.6 ± 3295.6	6762.7 ± 203.5	5562.0 ± 468.1	34214.6 ± 1563.3
Hydrocinnamic acid (1)	1321	ND	436.0 ± 50.1	ND	171004. ± 9358.5	85320.6 ± 304.7	259394.9 ± 4645.0
Lupeol (2)	4071	14413.9 ± 231.1	43925.0 ± 490.9	29510.2 ± 523.7	6075.4 ± 81.5	ND	37853.0 ± 488.5
Methyl cis-9-octadecenoate (1)	2109	20049. ± 683.1	1100.2 ± 31.5	45610.6 ± 525.8	7540.9 ± 99.3	8756.8 ± 316.7	25537.9 ± 323.9
Methyl hexadecanoate (1)	1929	12438.1 ± 337.9	4725.6 ± 72.9	24270.1 ± 1908.9	13592.1 ± 544.3	7922.5 ± 830.0	72900.8 ± 2824.6
Methyl hydrocinnamate (1)	1269	351.5 ± 24.3	506.6 ± 40.5	361.9 ± 13.9	47380.7 ± 3112.0	74837.1 ± 2401.6	201678.7 ± 3523.5
Methyl retinoate (1)	2544	ND	ND	ND	19459.5 ± 252.1	11410.4 ± 309.3	628.4 ± 11.9
Methyl tetracosanoate (2)	2713	13296.3 ± 2467.2	1635.6 ± 107.6	31887.1 ± 3402.2	8741.7 ± 847.3	1415.9 ± 218.7	35976.1 ± 2437.9
Procerin (2)	1937	ND	ND	ND	5940.0 ± 245.9	73599.8 ± 2554.3	3442.1 ± 44.8
Spathulenol (2)	1595	257.6 ± 20.0	ND	466.4 ± 17.1	6337.4 ± 35.5	2869.0 ± 29.8	27413.7 ± 152.4
Stigmasterol (1)	3769	ND	ND	ND	978.96 ± 44.9	ND	6932.3 ± 141.6
Tetradecanal (2)	1609	546.4 ± 24.1	513.0 ± 43.2	608.0 ± 6.8	4088.9 ± 128.3	948.7 ± 18.7	29637.4 ± 166.4
Trans-nerolidol (1)	1557	821.8 ± 31.5	437.4 ± 13.7	1474.6 ± 81.0	31946.5 ± 314.1	4371.4 ± 31.7	20447.5 ± 266.8
1-Octacosanol (2)	3183	16683.7 ± 1124.2	ND	48724.7 ± 307.6	3936.0 ± 49.3	ND	21199.6 ± 332.0
5-(2,5-Dimethylphenyl-2(3H)-furanone (2)	1630	ND	ND	ND	160349. ± 7486.7	141050.2 ± 4171.7	14490.3 ± 101.1

Levels of identification are presented in parentheses according to Sumner et al. (2007). (1) Identified compounds (chemical reference standards). (2) Putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries). BPE (brown propolis ethanol), BPH (brown propolis hexane), BPD (brown propolis dichloromethane), GPE (green propolis ethanol), GPH (green propolis hexane), GPD (green propolis dichloromethane). ND = not detected. All analyses were performed using three replicates.

3. Results and discussion

3.1. Overall variation in metabolite composition of propolis samples

Metabolite profiling of green and brown Brazilian propolis extracts was performed by employing a GC–MS approach in order to assess their chemical composition. More than one hundred peaks were identified of which 29 were successfully annotated. We could detect some sesquiterpenes (e.g. δ-cadinene, caryophyllene, caryophyllene oxide, etc.), diterpenes (epimanool), triterpenes (lupeol), fatty acids esters (e.g. ethyl hexadecanoate, methyl tetracosanoate, methyl hexadecanoate, etc.) and many others metabolites (Table 1). Thirty-four metabolites were classified according to their MS spectral similarity to known compounds of a given chemical class, as follows: triterpenoids (12), acetyltriterpenoids (3), sesquiterpenes (6), steroids (4) and hydrocarbons (9) (Supplemental Table 1). Thirty-two metabolites were exclusively detected in green propolis extracts (Table 1 and Supplemental Table 1), including caryophyllene oxide, methyl retinoate, stigmasterol, two sesquiterpenes, one steroid and one triterpene, whereas only twelve metabolites were exclusively found in brown propolis extracts, but mostly unidentified compounds.

In general, higher levels of sesquiterpenes and steroids were found in green propolis samples, whereas triterpenoids were present in higher quantities in brown propolis samples (Table 1, Supplemental Table 1). For example, nerolidol, δ-cadinene, γ-muurolene, and caryophyllene were detected in both green and brown propolis extracts, whereas caryophyllene oxide was only detected in green propolis samples. After liquid–liquid extraction, high levels of these sesquiterpenes were found in GPH and BPH. Nerolidol, δ-cadinene, γ-muurolene, caryophyllene and its derivative caryophyllene oxide are naturally occurring sesquiterpenes found in the essential oils of many types of plants (Alves et al., 2013; Ho, Liao, & Su, 2012; Senatore et al., 2013), but also in green propolis samples (De Albuquerque, Alves, Lemos, Dorneles, & De Morais, 2008; Torres, Lopes, Neto, & Citó, 2008). The chemical composition of the essential oil obtained by hydrodistillation of green propolis has been analyzed by GC-FID and GC/MS (De Albuquerque et al., 2008). Seventeen compounds, mainly mono and sesquiterpenes, were identified representing 91.0% of compounds presents in the oil. Five out of these 17 metabolites were also identified in our study: caryophyllene, caryophyllene oxide, γ-muurolene, trans-nerolidol, and spathulenol (Table 1). Guimarães et al. (2012) investigated the chemical composition of green propolis extracts prepared by fractionated percolation using propylene glycol as solvent. Only five compounds were detected: caffeic acid, p-coumaric acid, cinnamic acid, aromadendrin, and isosakuranetin. None of these compounds were, however, detected in our study. Tavares et al. (2010) reported that the phytochemical investigation of Brazilian green propolis extracts prepared by maceration in ethanol, led to the isolation of nine compounds: α- and β-amyrin, lupeol, ramnocitrin, eupalitin, acacetin, 3-prenyl-4-hydroxycinnamic acid, 3,5-diprenyl-4-hydroxycinnamic acid, and (E)-3-[4-(3-phenylpropanoiloxy)]-3,5-diprenil-cinnamic acid. From those compounds only lupeol was also detected in our study. GPH and BPH contained high levels of most of the identified fatty acids, mainly because of the lipophilic properties of the solvent (hexane) (Table 1). Fatty acids are compounds commonly found in propolis samples (Castro et al., 2009; Cheng et al., 2013; Cheng et al., 2015; Costa Da Silva et al., 2013; Duran et al., 2011).

Taken together, these results show that the choice of extraction method has a strong effect the chemical composition of the extracts.

To provide comparative interpretations for the changes in the metabolite composition of the different propolis samples, a series of uni- and multivariate analyses were employed. ANOVA was performed to assess the overall variation in metabolite levels, followed by post-hoc analyses (Bonferroni correction, False Discovery Rate < 0.05). Principal component analyses (PCA) was applied aiming at finding the directions that best explain the variance in the data set. Principal component 1 explained 51% of the total variance, whereas principal component 2 explained 24% of the total variance (Fig. 1a). The PCA plot clearly differentiated propolis samples derived from green and brown propolis. GPD, GPE and GPH clustered together at the upper left side of the plot, whereas BPD, BPE and BPH clustered at the bottom right corner. Curiously, BPD samples were positioned much closer to the green propolis samples than to BPE and BPH, suggesting that the chemical composition of BPD is more similar to the green propolis samples than BPE and BPH. By performing hierarchical cluster analysis of all detected metabolites in the propolis extracts, samples were divided into two main groups: the first one containing all green propolis extracts (GPD, GPE and GPH) and BPD, whereas the second one comprised of BPE and BPH. These results confirm the closeness of the metabolomes of BPD and all green propolis extracts (Figs. 1b and 2).

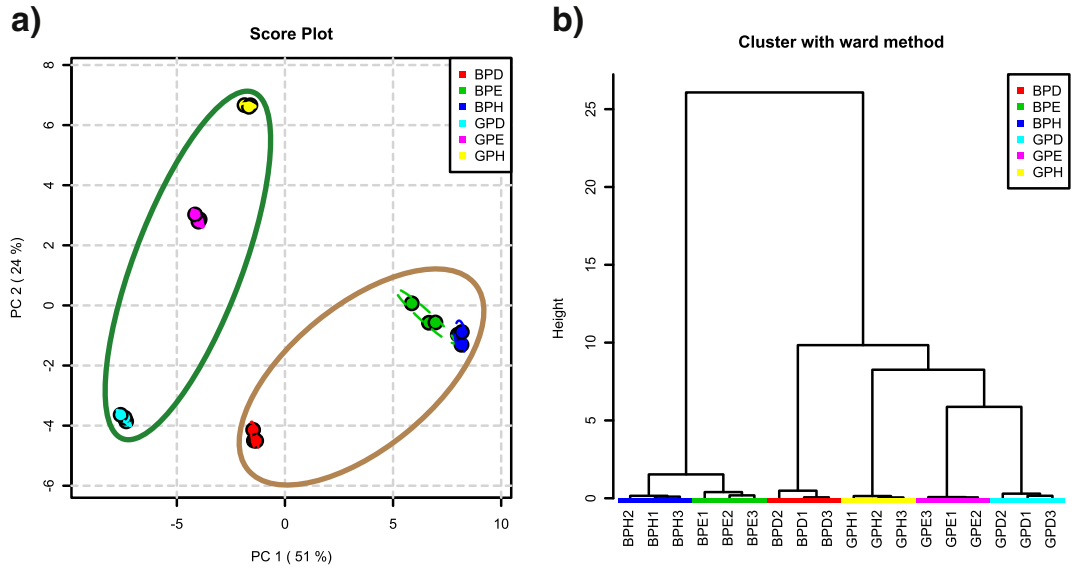

Fig. 1. (a) Principal component analysis (PCA) of green and brown Brazilian propolis samples based on the GC–MS profile. (b) Dendrogram resulting from a hierarchal cluster analysis based on the distribution of the 64 detected metabolites. BPE (brown propolis ethanol), BPH (brown propolis hexane), BPD (brown propolis dichloromethane), GPE (green propolis ethanol), GPH (green propolis hexane), GPD (green propolis dichloromethane).

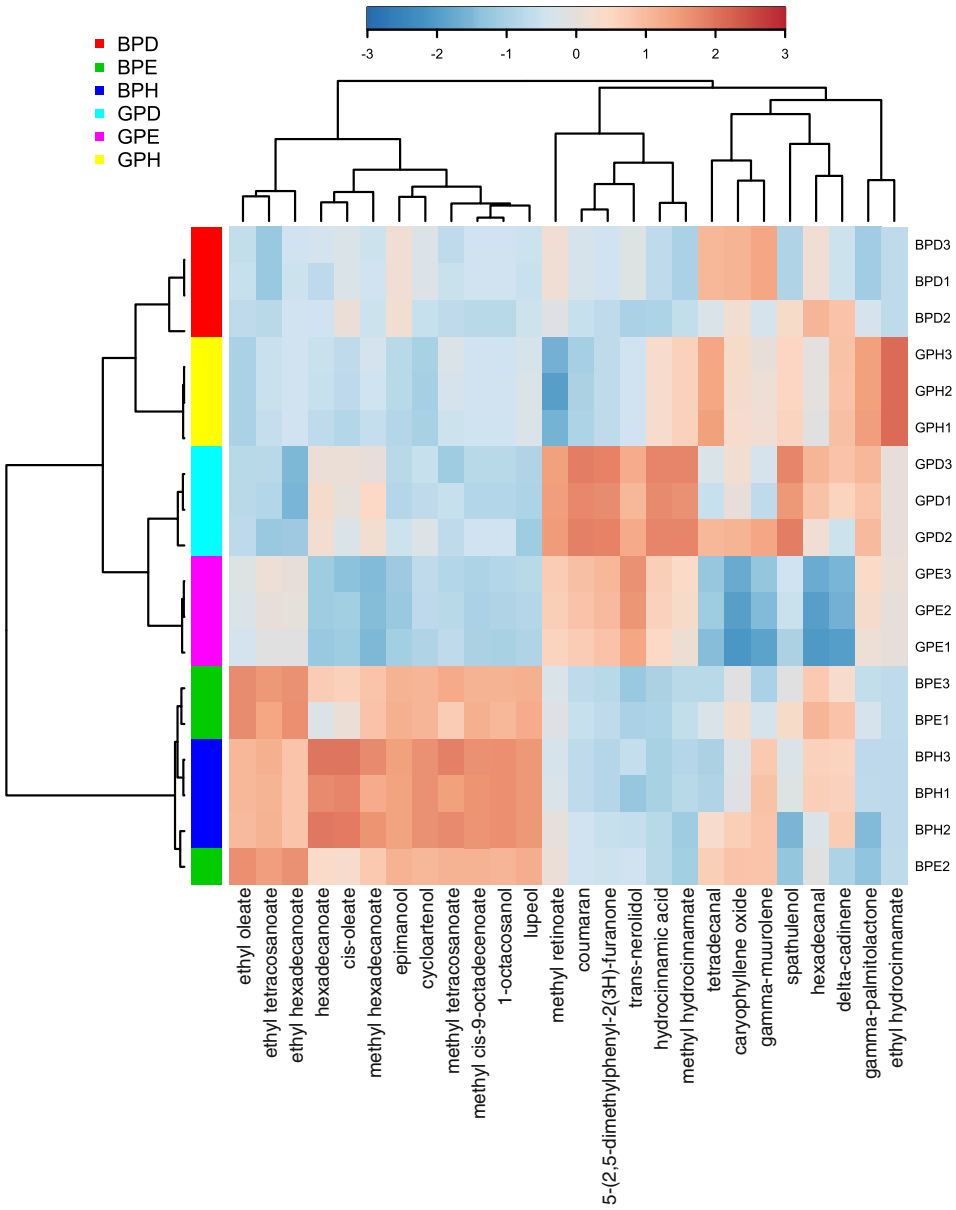

Fig. 2. Heatmap representation of the metabolite-metabolite correlations in green and brown Brazilian propolis samples. Correlations coefficients were calculated based on Pearson's correlation. BPE (brown propolis ethanol), BPH (brown propolis hexane), BPD (brown propolis dichloromethane), GPE (green propolis ethanol), GPH (green propolis hexane), GPD (green propolis dichloromethane).

In the PCA analysis samples are clustered based on their similarities and differences, by reducing the number of dimensions without much loss of information. This analysis can be used for a myriad of applications. For example, GC–MS analysis and electronic nose combined with PCA was successfully applied to distinguish the geographical origin of twelve propolis samples from 4 different regions of China (Cheng et al., 2013). In a different study, headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry and olfactometry (GC–MS–O) were used to characterize the aroma-active profiles of the fruits from three different bayberry cultivars. PCA of the active aromas revealed their contributions to the odor differences among the bayberry cultivar groups (Cheng et al., 2015). PCA was also used to assess potential relationships between major cocoa components (catechins and methylxanthines) and geographical origin in cocoa beans from different cocoa-growing areas of Colombia (Carrillo, Londoño-Londoño, & Gil, 2014). Taken together, these results demonstrate the versatility and usefulness of the combination of chromatographic analysis and PCA to obtain further biological meaning.

3.2. Antioxidant capacity and total phenolic content

The DPPH radical scavenging assay is based on the reactivity of the DPPH radical towards the hydrogen-donors molecules presented in the extracts. A higher DPPH radical scavenging activity is associated with a lower IC_{50}, which represents the concentration of the extract necessary to decrease the initial absorbance of the DPPH solution by 50%. The DPPH radical scavenging activity of the fractions ranged from 21.50 to 78.77 μg/mL and depended on the type of solvent used for the extraction. The strongest antioxidant activities (lower IC_{50}) were found in GPE (28.72 ± 0.38), GPD (21.50 ± 0.32) and BPD (31.72 ± 0.33). The IC_{50} observed for BPD (31.72 ± 0.33) is almost half of the value observed for BPE (60.53 ± 0.47), whereas the IC_{50} observed for GPD (21.50 ± 0.32) is almost three-fourths of the value

observed for GPE (28.72 ± 0.38) (Table 2). Therefore, partition in dichloromethane enhanced the extraction of antioxidant compounds as we can infer from the lower values of IC_{50} observed for GPD and BPD as compared with GPE and BPE. Partition in hexane, however, decreased the antioxidant capacity of green propolis extracts. The IC_{50} observed for GPH (78.77 ± 1.22) is 2.7-fold higher than the IC_{50} observed for GPE (28.72 ± 0.38) (Table 2). GPH has, therefore, lower antioxidant capacity than GPE and GPD.

These results are in agreement with the findings of Marimuthu, Wu, Chang, and Chang (2008) who tested the antioxidant activity of extracts obtained from *Chamaecyparis obtusa* and showed that the antioxidant activity of ethanolic extracts were 2-fold higher than the antioxidant activity of the extracts obtained by partition with hexane (Marimuthu et al., 2008).

By definition, an antioxidant is a molecule that inhibits the oxidation of other molecules. The protection provided by any antioxidant compound depends on its concentration, but mainly on its reactivity towards the particular reactive oxygen species being considered. Antioxidant capacity is usually closely associated with the content of phenolic compounds which may exert a synergetic effect between themselves and with some lipophilic compounds such as vitamins C and E. Phenolic compounds are presumed to be good antioxidant molecules due to their extensive conjugated π-electron systems that facilitates the donation of electrons from the hydroxyl moieties to oxidizing radical species.

One disadvantage of gas chromatography is the fact that samples must be volatile or volatilized by mean of derivatization, since their components will be separated in the gas phase. However, some compounds might not be detected by GC–MS even after the samples are derivatized due to their high molecular weight. In this context, we measured the content of total phenolics in the propolis extracts. Total phenolic content of the green propolis ranged from 38.85 to 204.30 mg GAE/g of dry weight, whereas for the brown propolis it ranged from 31.88 to 62.48 mg GAE/g of dry weight (Table 2). The highest levels of phenolics compounds were found in GPE (185.52 ± 1.09 mg GAE/g of dry weight) and GPD (204.30 ± 3.80 mg GAE/g of dry weight). Although the content of phenolic compounds in brown propolis was in general lower than in green propolis, a similar trend was observed: total phenolic content was higher in BPD (62.48 ± 0.61 mg GAE/g of dry weight) than in BPE (48.24 ± 1.09 mg GAE/g of dry weight). Partition in dichloromethane, therefore, enhanced the levels of phenolic compounds in the fractions GPD and BPD. Partition in hexane, however, decreased the levels of phenolic compounds in BPH (31.88 ± 0.61 mg GAE/g of dry weight) and GPH (38.85 ± 0.80 mg GAE/g of dry weight) as compared with BPE and GPE, respectively. These results are in agreement with the findings of Önder, Ay, and Sarker (2013) who tested the effect of solvents with different polarity on the extraction of total phenolic compounds from *Humulus lupulus*. They showed that the total phenolic content of 25% aqueous ethanol extracts were 6.7-fold higher than the total phenolic content of the extracts obtained with hexane.

3.3. Antimicrobial activity

Antibacterial activities of the fractions were assessed against the Gram-positive bacteria *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), and *M. luteus* (ATCC 10240) (Table 3). Since propolis is most frequently administered orally, these bacteria were selected because of their involvement in diseases related to the gastrointestinal and respiratory tracts. In general, the minimum inhibitory concentrations (MICs) of brown propolis extracts were lower than the MICs of green propolis extracts. For example, the MIC against *B. subtilis* was 31.25 μg/mL for BPE, 15.62 μg/mL for BPD, and 31.25 μg/mL for BPH. These values are lower than those observer for GPE (125 μg/mL), GPD (62.5 μg/mL), and GPH (>500 μg/mL). The same trend was observed against *S. aureus* and *M. luteus*. The only exception was observed against *M. luteus* in which the MIC for BPE (250 μg/mL) was greater than the MIC for GPE (125 μg/mL). These results indicate, therefore, that brown propolis samples are more effectively inhibiting the growth of all bacteria under the tested conditions. BPD and GPD showed the most powerful activity among all brown and green propolis extracts. Unlikely of what was observed in relation with antioxidant properties, partition in hexane has enhanced or, at least, kept the original activity of the brown propolis extracts. GPH, however, showed no activity (MIC > 500 μg/mL) against *S. aureus* and *B. subtilis*. These results support our previous observation that partition in dichloromethane enhances the extraction of bioactive compounds. The MIC of ethanolic and aqueous extracts of green propolis was determined against isolates of *Staphylococcus* spp. These extracts showed MIC of 50 μg/mL, which is indicative of a strong in vitro activity (Santos Neto et al., 2009). Farnesi, Aquino-Ferreira, De Jong, Bastos, and Soares (2009) examined the antibacterial activities of several types of propolis, including green propolis, against four species of bacteria via bioautography. The antibacterial activity against *S. aureus* was tested in only one concentration of green propolis and, therefore, no MIC was reported.

In this study, the extracts tested showed higher antibacterial activity than those reported for Brazilian propolis against various Gram-positive anaerobic microorganisms (Santos et al., 2002). To the best of our knowledge, this is the first study that presents a detailed analysis of the antibacterial activity of brown and green propolis extracts obtained from different extracts against *S. aureus*, *B. subtilis*, and *M. luteus*, combining with correlation and multivariate analyses to identify potential bioactive compounds.

3.4. Use of correlation and multivariate statistical analyses to identify potential bioactive compounds

In an attempt to identify potential compounds that contribute to the biological activity of the extracts, a correlation analysis was performed

Table 2
Antioxidant capacity and total phenolics of green and brown Brazilian propolis samples.

Tested sample	Antioxidant capacity (IC_{50}, μg/mL)	Total phenolics (mg GAE/g of dry weight)
BPE	60.53 ± 0.47 (d)	48.24 ± 1.09 (c)
BPD	31.72 ± 0.33 (c)	62.48 ± 0.61 (d)
BPH	60.37 ± 0.18 (d)	31.88 ± 0.61 (a)
GPE	28.72 ± 0.38 (b)	185.52 ± 1.09 (e)
GPD	21.50 ± 0.32 (a)	204.30 ± 3.80 (f)
GPH	78.77 ± 1.22 (e)	38.85 ± 0.80 (b)
Galic acid	1.26 ± 0.02	–

BPE (brown propolis ethanol), BPH (brown propolis hexane), BPD (brown propolis dichloromethane), GPE (green propolis ethanol), GPH (green propolis hexane), GPD (green propolis dichloromethane). Different letters in parentheses indicate significant differences between samples by Tukey's HSD ($P < 0.05$). All analyses were performed using three replicates.

Table 3
Antibacterial activity of green and brown Brazilian propolis samples.

Tested sample	*B. subtilis* (ATCC 6633) (μg/mL)	*M. luteus* (ATCC 10240) (μg/mL)	*S. aureus* (ATCC 6538) (μg/mL)
BPE	31.25	250	15.62
BPD	15.62	7.81	15.62
BPH	31.25	7.81	15.62
GPE	125	125	500
GPD	62.5	62.5	125
GPH	>500	500	>500
Chloramphenicol	3.12	1.56	6.25

BPE (brown propolis ethanol), BPH (brown propolis hexane), BPD (brown propolis dichloromethane), GPE (green propolis ethanol), GPH (green propolis hexane), GPD (green propolis dichloromethane). All analyses were performed using three replicates.

between the IC_{50} and MIC values and the relative content of all identified metabolites, including the total phenolic compounds, presented in the fractions (Fig. 3). Lower IC_{50} and MIC values are associated with higher antioxidant and antibacterial activities the extracts, thus, to allow a more direct comparison it was used as input for this analysis the inverse of the IC_{50} and MIC values (Fig. 3). A threshold of 0.5 was set in order to consider the correlation as strong.

Total phenolic compounds and methyl retinoate showed a strong positive correlation with the antioxidant capacity (Fig. 3a). Methyl retinoate was only detected in green propolis samples (Table 1), whereas the highest content of total phenolic compounds was found for GPD and GPE. Both phenolic compounds and methyl retinoate possess an extensive conjugated π-electron system that facilitates the donation of electrons to oxidizing radical species. In contrast, tetradecanal, γ-palmitolactone and ethyl hydrocinnamate showed a strong negative correlation with the antioxidant capacity. The lowest antioxidant activities were found for GPH and BPH which possess high levels of these three compounds. Taken together, these results support the fact that GPD and GPE are the fractions with the highest antioxidant capacity (Table 2). Several studies have reported the antioxidant properties of the phenolic compounds (Avello, Pastene, Bustos, Bittner, & Becerra, 2013; Pereira et al., 2014; Wang et al., 2013). A recent study has evaluated the antioxidant capacity of free retinoic acid by four different scavenging assays. The results showed significant scavenging activities in all these antioxidant assays comparable to the standard antioxidant (Siddikuzzaman & Grace, 2013). Although a high correlation between phenolic compounds and antioxidant capacity is expected, these results highlight the robustness of our approach to identify potential bioactive compounds.

Different sets of metabolites are shown to correlate with the antibacterial activity of the extracts which is largely dependent on the tested microorganism (Fig. 3b–d). Methyl retinoate showed a strong positive correlation with the antibacterial activity against *B. subtilis*, whereas γ-palmitolactone and ethyl hydrocinnamate showed a strong negative correlation with the antibacterial activity against *B. subtilis* and *M. luteus*. Tetradecanal, γ-palmitolactone and ethyl hydrocinnamate

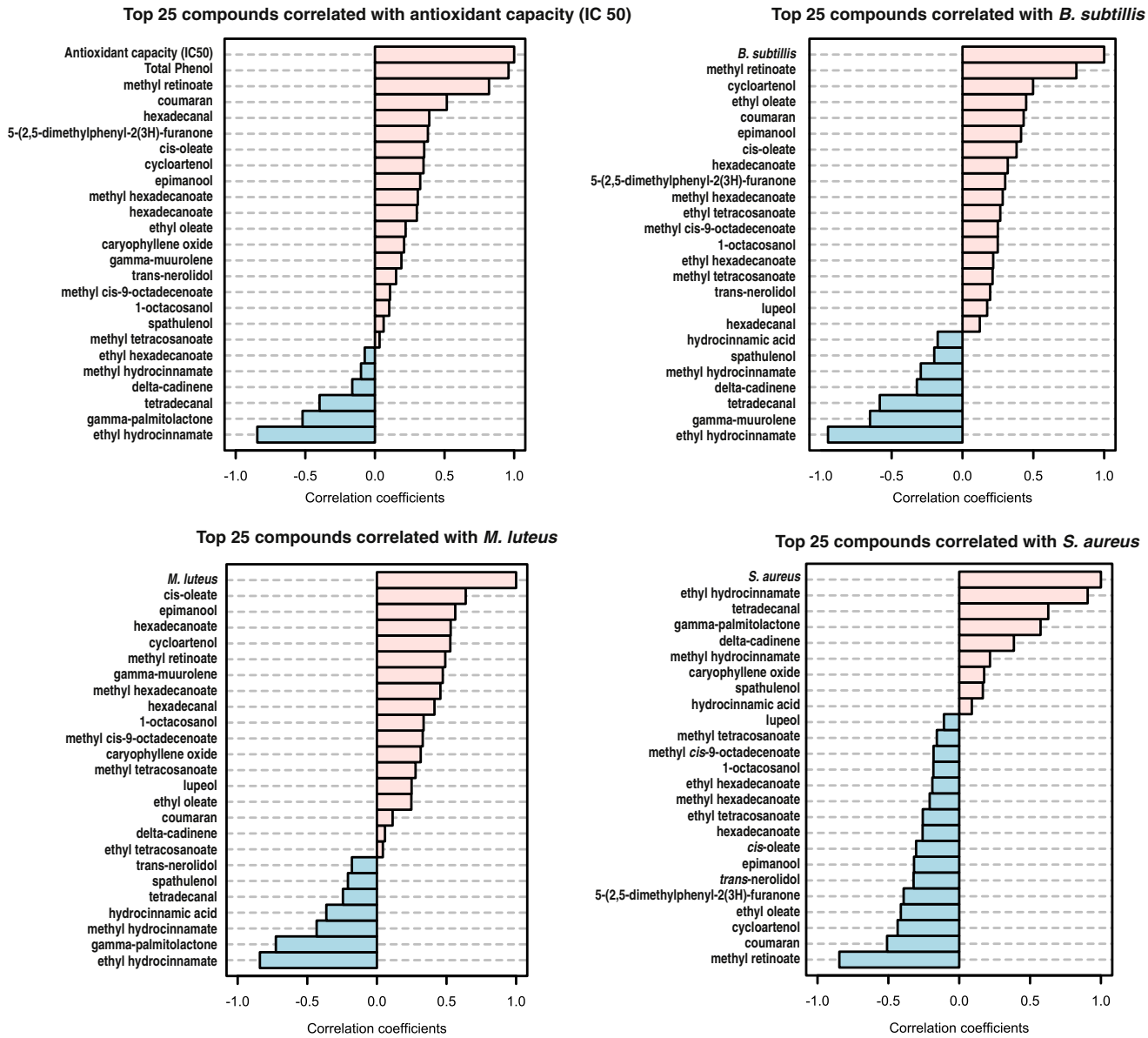

Fig. 3. Top 25 metabolites identified in brown and green Brazilian propolis samples which correlated with the (a) antioxidant capacity and antibacterial activity against (b) *B. subtillis*, (c) *M. luteus* and (d) *S. aureus*. The light blue bars show those metabolites showing a negative correlation and the light pink bars show those with a positive correlation with the given property.

showed a strong positive correlation with the antibacterial activity against *S. aureus*, whereas methyl retinoate showed a strong negative correlation with the antibacterial activity against *S. aureus*. No strong positive correlations were found for *M. luteus*, suggesting that there is a nonlinear concentration-dependent inhibition of this microorganism, which could be explained by the ability of the detected bioactive compounds to act synergistically. These results indicate that the mechanism(s) underlying the antimicrobial activity against these microorganisms encompass a complex trait, which might involve synergistic effects among the metabolites present in the extracts.

4. Conclusions

Green and brown Brazilian propolis were chemically analyzed and their antioxidant and antibacterial activities determined. Twenty-nine metabolites were identified while 34 other metabolites were classified according to their MS spectral similarity to known compounds of a chemical class. Thirty-two metabolites were exclusively detected in green propolis, while only 12 metabolites were exclusively found in brown propolis extracts, mostly unidentified compounds. In general, partitioning in dichloromethane has enhanced the extraction of antioxidant and antibacterial compounds, as it can be inferred from the lower values of IC_{50} and MIC observed for GPD and BPD and it has also enhanced the levels of phenolic compounds in the fractions. The approach used allowed the identification of likely candidate compounds to explain the observed biological activity the extracts. These results also indicate that the mechanism underlying the antimicrobial activity against these microorganisms is a complex trait, which might involve synergistic effect between the metabolites presented in the extracts.

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