Journal of Pharmaceutical Analysis 7 (2017) 280–287

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa

Original Research Article

Chemical and microbiological characterization of tinctures and microcapsules loaded with Brazilian red propolis extract

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A R T I C L E I N F O

Keywords:
Red propolis
Tinctures
Microcapsules
Isoflavonoids
LC/ESI/FTMS/Orbitrap
Antioxidant activity
Antibacterial activity

A B S T R A C T

The aim of this study was to characterize tinctures and microcapsules loaded with an ethanol extract of red propolis through chemical, physicochemical and microbiological assays in order to establish quality control tools for nutraceutical preparations of red propolis. The markers (isoflavonoids, chalcones, pterocarpans, flavones, phenolic acids, terpenes and guttiferones) present in the tinctures A and B were identified and confirmed using LC/ESI/FTMS/Orbitrap. Four compositions (A, B, C and D) were prepared to contain B tincture of the red propolis with some pharmaceutical excipients and submitted to two drying processes, i. e. spray-drying and freeze-drying to obtain microcapsules loaded with the red propolis extract. The tinctures and microcapsules of the red propolis were submitted to the total flavonoid content and antioxidant activity tests. The antibacterial activity and minimum inhibitory concentration (MIC) were tested using *Staphylococcus aureus* ATCC 25293 and *Pseudomonas aeruginosa* ATCC 27853 strains. The tinctures and microcapsules presented high flavonoid quantities from 20.50 to 40.79 mg/100 mg of the microcapsules. The antioxidant activity and IC_{50} were determined for the tinctures A and B (IC_{50}: 6.95 µg/mL and 7.48 µg/mL), the spray-dried microcapsules (IC50: 8.89–15.63 µg/mL) and the freeze-dried microcapsules (IC50: 11.83–23.36 µg/mL). The tinctures and microcapsules were proved to be bioactive against gram-positive and gram-negative bacteria with inhibition halos superior to 10 mm at concentration of 200 µg/mL and MIC values of 135.87–271.74 µg/mL using gram-positive strain and 271.74–543.48 µg/mL using gram-negative strain. The tinctures and microcapsules of the red propolis have a potential application for nutraceutical products.

1. Introduction

The *Apis mellifera* bee species collects resins and exudates from plants and adds their salivary secretions to produce propolis, which is a biotechnological product with several biological activities. Propolis has been widely used as an alternative and traditional medicine to treat several diseases [1]. The Brazilian propolis is classified into 13 types (groups) according to its chemical and physicochemical properties [2]. The red propolis is incorporated into Group 13 and can be found in the northeastern states of Brazil (e.g. Bahia, Paraiba, Sergipe, Pernambuco and Alagoas) [3]. The chemical composition of the red propolis has been investigated and the presence of isoflavones [2], chalcones [4], isoflavans, flavonols, pterocarpans [5], terpenes and polyisoprenylated benzophenones [6] has been identified.

The red propolis produced in the state of Alagoas, Brazil, received a seal of geographical indication (appellation of origin) because of its distinct biological activities and standardized production process involving the working communities and a scientific research program with different and on-going governmental and non-governmental actions. The Brazilian apiceutical industries are developing microcapsules of propolis, which after being processed, result in an intermediate product in which the concentration of flavonoids has been standardized

Peer review under responsibility of Xi'an Jiaotong University.

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http://dx.doi.org/10.1016/j.jpha.2017.03.004
Received 5 May 2016; Received in revised form 22 January 2017; Accepted 17 March 2017
Available online 18 March 2017
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through the development of new chemical and physicochemical methods and microbiological assays to ensure its quality. The development of new release systems for apical products in the form of microcapsules is of utmost importance to ensure stability of the composition, facilitate administration, facilitate the release of the active substances of the pharmaceutical form, as well as the bioavailability of the isoflavonoids present in these apiceutical products.

The UV/Vis spectrophotometry is a simple, low-cost and alternative analytical method for quality assurance of propolis and its bioproducts. The UV/Vis spectrophotometry can be used for determination of total flavonoids and phenolic compounds using derivatization reactions with chelating agents or by direct reading using an appropriate standard [7,8]. High performance liquid chromatography coupled to different detectors as UV/visible detector, diode-array detector and liquid chromatography coupled to mass spectrometry detector (HPLC/UV, HPLC/DAD, and LC/MS) and gas chromatography coupled to mass spectrometry (GC/MS) are widely used in the complex analysis of foods, beverages, pharmaceuticals and cosmetics. HPLC/UV and LC/MS are good strategies to analyze flavonoids and phenolic compounds and terpenes, especially by determining the compounds present in the propolis and other bee products [9–11].

Some biological methods are used to evaluate the effectiveness of propolis. Bioassays against *Artemia salina* [12] and *Saccharomyces cerevisiae Pdr5p* [13] are cited, but the microbiological methods are more trustable, which have specifically been used as agar diffusion and microdilution methods for the determination of the minimum inhibitory concentration (MIC). The microdilution method is cheap, reproducible and highly sensitive, requires a smaller amount of samples and also allows the permanent record [14]. The aim of this paper was to characterize the tinctures and microcapsules loaded with red propolis extracts through chemical, physicochemical and microbiological assays in order to establish the quality control tools for the nutraceutical preparations of the red propolis.

2. Experimental

2.1. Chemicals and biologicals

The flavonoids, namely, chrysin, catechin, pinocembrin, kaempferol, daidzein, genistein, naringenin, galangin, formononetin, biochanin A, catechin, caffeic acid, ferulic acid and p-coumaric acid, were acquired from Sigma-Aldrich (St. Louis, MO. USA). Epicatechin and liquiritigenin were acquired from Extrasynthese® (Lyon Nord, France) and were used as analytical standards. The flavonoids, namely, quercetin, luteolin, rutin, were obtained as secondary standards at the Pharmacy Department of the Federal University of the Rio Grande do Norte, Brazil.

Analytical grade reagents included ethanol, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and formic acid. HPLC grade methanol was purchased from J.T. Baker (Mallinckrodt, Mexico), and acetonitrile was purchased from Fisher Scientific (Leicestershire, UK) and the Milli-Q grade water was produced in a lab.

The bacteria strains were from the American Type Culture Collection (ATCC), *Staphylococcus aureus* (ATCC 25293) and *Pseudomonas aeruginosa* (ATCC 27853). Mueller Hinton agar, BHI agar, nutrient agar and resazurin were used as culture media for the microbiological testings.

2.2. Red propolis

Red propolis raw material (300 g) was collected in April 2012, from Marechal Deodoro-Alagoas, Brazil, the apiary Ilha do Porto (Propolis A) with geographical coordinates of the south latitude: 9° 44.555´, the west latitude: 35° 52.080´ and the height of 18.1 m, and apiary Primavera (Propolis B) with geographical coordinates of the south latitude: 9° 42.258´, the west latitude: 35° 54.391´ and the height of 35.5 m. The access and transportation of the red propolis were previously authorized by the regulatory agency (CNPq, under the protocol number of acceptance 010124/2012-8) to comply with the Brazilian legislation for the Genetic Heritage and biodiversity conservation.

2.3. Crude extract preparation

The raw propolis in nature (250 g) was used to obtain the active constituents using the extraction method through maceration with 80% ethanol (600 mL). At the end of 3 cycles of extraction, the crude extract was concentrated using a rotary evaporator (Fisatom) and 100 g of a crude extract of propolis was obtained. The dry mass was stored in a freezer at −20 °C until further analysis and used to prepare the tinctures A and B as well the chloroform extracts (A and B), which were obtained using liquid-liquid extraction.

2.4. Preparation of microcapsules loaded with red propolis extract

Four compositions (A, B, C and D) were prepared to contain B tincture of red propolis with some variations in proportions of excipients used: guar gum, pectin, maltodextrin, carbapol, carboxymethylcellulose, stearic acid and colloidal silicon dioxide (Table 1). The compositions were submitted to two drying processes, i.e. spray-drying and freeze-drying, to obtain 8 formulations of microcapsules, which were previously characterized by scanning electron microscopy (SEM). The microcapsules loaded with red propolis extract (MRPE) obtained through spray-drying (MRPE A-SD, MRPE B-SD, MRPE C-SD and MRPE D-SD) and freeze-drying (MRPE A-FD, MRPE B-FD, MRPE C-FD and MRPE D-FD) were submitted to the total flavonoid content, antioxidant activity, antimicrobial activity and MIC tests.

2.5. Determination of the propolis markers using HPLC/UV and LC/ESI/FTMS/Orbitrap

The identification of the red propolis markers in the tinctures and microcapsules was performed using high performance liquid chromatography coupled to a diode array detector (HPLC/DAD) (Shimadzu, Tokyo, Japan). The propolis tinctures and microcapsules were prepared in a concentration of 100 mg/mL using absolute ethanol as solvent and then diluted to a concentration of 1.0 mg/mL.

The HPLC/DAD system consisted of an LC-20ADXR pump, a CTO-10AD oven, a UV SDM-20A detector, SIL-20AXR auto-injector and a CMB-20A controller coupled to a computer through a Lab-Solution software from Shimadzu. The mobile phase consisted of a gradient system: 0.1% of formic acid in Milli-Q water (A) and HPLC grade methanol (B). The separation was achieved using a Phenomenex C_{18} column (250 mm×4.6 mm i.d., 5 µm) fitted with a Phenomenex security guard C_{18} column (4.0 mm×3.0 mm i.d., 5 µm) and maintained at a temperature of 33 °C. The flow rate was 0.80 mL/min and

Table 1
Compositions of different microcapsules of red propolis extract submitted to spray-drying and freeze-drying processes.

Components	Compositions (%)			
	MRPE A	MRPE B	MRPE C	MRPE D
Propolis extract	75.00	47.62	76.93	64.94
Guar gum	7.50	31.74	7.69	1.30
Maltodextrin	5.00	–	5.13	–
Carbapol	7.50	7.94	7.69	–
Carboxymethylcellulose	–	–	–	5.19
Pectin	–	7.94	–	23.38
Stearic acid	2.50	–	–	–
Colloidal silicon dioxide	2.50	4.76	2.56	5.19
Total	100.00	100.00	100.00	100.00

the discrete channel on the UV detector was set to acquire data at 281 nm. A gradient system was programmed: 30% of solvent B in the range between 0 and 7 min, 40% of solvent B in 15 min, 45% of solvent B in 30 min, 60% of solvent B in 40 min, 80% of solvent B in 50 min, 90% of solvent B in 60 min, and then solvent B was reduced to 30% in 65 min and remained in this isocratic condition for 70 min. The samples were introduced in HPLC using a Rheodyne injector with the injection volume of 20 μL.

The identification and confirmation of the red propolis markers only in the tincture used for the microcapsule preparation were performed using LC/ESI/FTMS/Orbitrap. The LC/ESI/FTMS/Orbitrap (Thermo Fisher Scientific, Hemel Hempstead, UK) was used in the following conditions: the stationary phase being a C_{18} column from ACE® (Advanced Chromatography Technologies, Abardeen, UK) (100 mm×4.6 mm i.d, 5 μm) and the flow rate being 300 μL/min. The mobile phase consisted of a gradient system: 0.1% formic acid in water (A) and 0.1% of formic acid in acetonitrile (B) (v/v). The column was eluted in gradient mode as follows: 0 min 30% of B, 6 min 45% of B, 10 min 60% of B, 14 min 75% of B, 18 min 90% of B, 22 min 100% of B, 22–47 min 100% of B, and decreased to 30% of B in 52 min, then held at 30% of B in 52–58 min. The FTMS was set to acquire ions in a negative mode with a needle voltage of 4.0 kV and sheath (50) and auxiliary (10) gas flows and arbitrary units. The instrument was scanned over the range from 50 to 1200 amu. A volume of 10 μL was injected into the LC/ESI/FTMS/Orbitrap.

2.6. SEM analysis

SEM images were analyzed to confirm the morphology and approximate size of microparticles in the solid state. The lyophilized and spray-dried microcapsules were fixed on the stubs with double carbon tape and covered with a gold film during the metallization process, which was done using 10 mA for 7 min in a System Sanyu Electron, Quick Coater Model SC-701. SEM micrographs were from Shimadzu microscope (SSX-550 Superscan model) and small spherical, shape microparticles were observed for spray-dried microcapsules. The SEM micrographs of lyophilized microcapsules showed aggregate micro-particles similar to aggregate plate forms and an evident coalescence of the particles with an increase in the size of the lyophilized microcapsules was also observed.

2.7. Total flavonoid content test

The tincture and microcapsules were previously submitted to separate assays to dehydration in an infrared oven at 105 °C for 15 min. The tincture and microcapsules of red propolis containing 100 mg of crude extract of propolis were weighed and solubilized separately with ethanol 96° GL in a volumetric flask (10 mL) to obtain a concentration of 10 mg/mL. The readings were obtained with a UV spectrophotometer at 280 nm after the previous dilution of the sample to a concentration of 40 μg/mL. The assays were performed in triplicate and the total flavonoid content was determined based on the concentration of standard catechin.

2.8. Antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl reagent (DPPH˙)

Quantitative evaluation of the antioxidant activity of the red propolis extract and the microcapsules was performed to determinate the inhibition capacity of the free radical DPPH reagent. The red propolis extract and microcapsules were monitored by measuring the decrease in absorbance of the solutions in different concentrations and the absolute ethanol was used as placebo-controlled.

The red propolis extract and microcapsules were prepared in an initial concentration of 1.0 mg/mL using a solvent system ethanol:H_2O (7:3, v/v) and aided with a sonication bath. Then, the samples were diluted to achieve the final concentrations of 80.0, 25.0, 10.0, 5.0 and 2.5 μg/mL in 5.0 mL volumetric flasks. Then, 2.0 mL of 0.3 mM DPPH reagent was added to the 5.0 mL volumetric flasks containing the samples. The reaction was developed in the dark at room temperature (26 °C) over 30 min. The absorbance readings were performed at 518 nm with a spectrophotometer (Model UV-1700, Shimadzu, Kyoto, Japan) [15].

2.9. Antibacterial activity

The standardized tincture of the red propolis (10%) was prepared in a volumetric flask (10 mL) using ethanol at 96°GL at a concentration of 100,000 μg/mL. The microcapsules of red propolis were prepared in the same concentration as the tincture was. The working solutions were diluted in phosphate buffer solution (7.4 pH) to obtain concentrations of 100, 200, 400, 600, 800, 1000 and 2000 μg/mL, and assayed against the *Staphylococcus aureus* ATCC 25293 and *Pseudomonas aeruginosa* ATCC 27853 strains.

The antibacterial activity test was performed using the agar diffusion method [16]. The standardized strains were inoculated in petri dishes containing 20 mL Mueller-Hinton agar using sterile swabs. One hundred microliters of the working solution containing tincture, chloroform extract and microcapsules (tested separately) were transferred to wells of 8 mm in diameter, which were prepared manually with the help of sterile tips. The petri dishes were incubated at 36 °C for 24 h and the results were obtained by measuring the diameter of the halos formed around the wells.

2.10. Determination of the minimum inhibitory concentration (MIC)

The broth microdilution assay was used to determine the MIC, which was done using 96 wells microplates containing 100 μL/well of the Muller-Hinton broth and 100 μL/well of the test samples, following the procedure described by Clinical and Laboratory Standards Institute (CLSI) [17], with some modifications. The stock solutions of 100 mg/mL of the test samples were prepared and then diluted in the Muller-Hinton broth to 10,000 μg/mL. Serial dilutions (in the ratio of 1:2, v/v) were prepared in concentrations ranging from 50 to 2000 μg/mL in microplates. 30 μL of bacterial suspension, about 1.5 $\times 10^6$ CFU/mL, was added to the wells containing 100 μL of Muller-Hinton broth with different final concentrations of test samples (concentrations ranging from 50 to 2000 μg/mL). The results were observed after adding 40 μL of resazurin solution (100 μg/mL) and re-incubation at 36 °C for 2 h. Blue spots in the microplates showed no growth of *S. aureus* and *P. aeruginosa* bacteria and pink spots in the microplates showed bacterial growth. The serial dilution of ethanol (96°GL) in Muller-Hinton broth was performed as a placebo, and the Muller-Hinton broth was used as negative control. The MIC values were defined as the lowest antibacterial concentration that inhibited the bacterial growth. The MIC was tested three times.

3. Results and discussion

3.1. Determination of the propolis markers using HPLC/UV and LC/ESI/FTMS/Orbitrap

The HPLC/UV method presented many chromatographic peaks in the red propolis extract demonstrating the complexity of the analyses for this apiceutical raw material, but it was possible to obtain relative resolution between the peaks using the chromatographic method proposed and make adjustments to the new LC/ESI/FTMS/Orbitrap gradient method based on this first method. The HPLC/UV analysis demonstrated the presence of phenolic acids, flavanones, flavones, chalcone and isoflavones in the composition of the red propolis tinctures and extracts. The chromatographic peaks were identified in tinctures of the red propolis at the concentration of 1.0 mg/mL compared to the standards (Fig. 1).

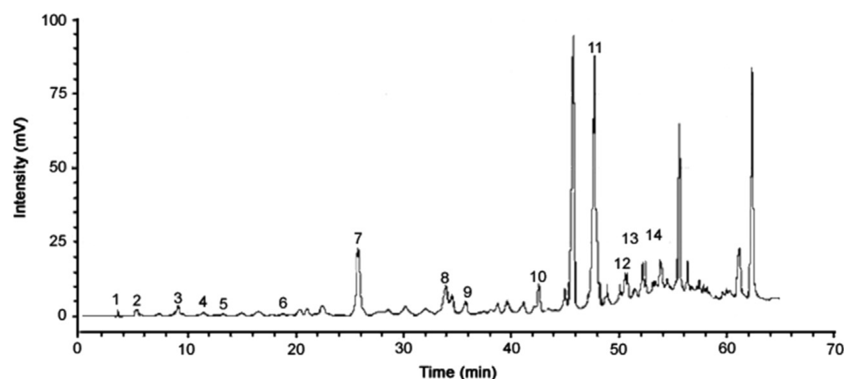

Fig. 1. Chromatographic profile of the red propolis tinctures at the concentration of 1.0 mg/mL. Flavonoids were identified using analytical standards: (1) catechin, (2) epicatechin, (3) caffeic acid, (4) p-coumaric acid, (5) ferulic acid, (6) rutin (7) liquiritigenin, (8) quercetin, (9), luteolin, (10) isoliquiritigenin, (11) formononetin, (12) pinocembrin, (13) biochanin A and (14) chrysin.

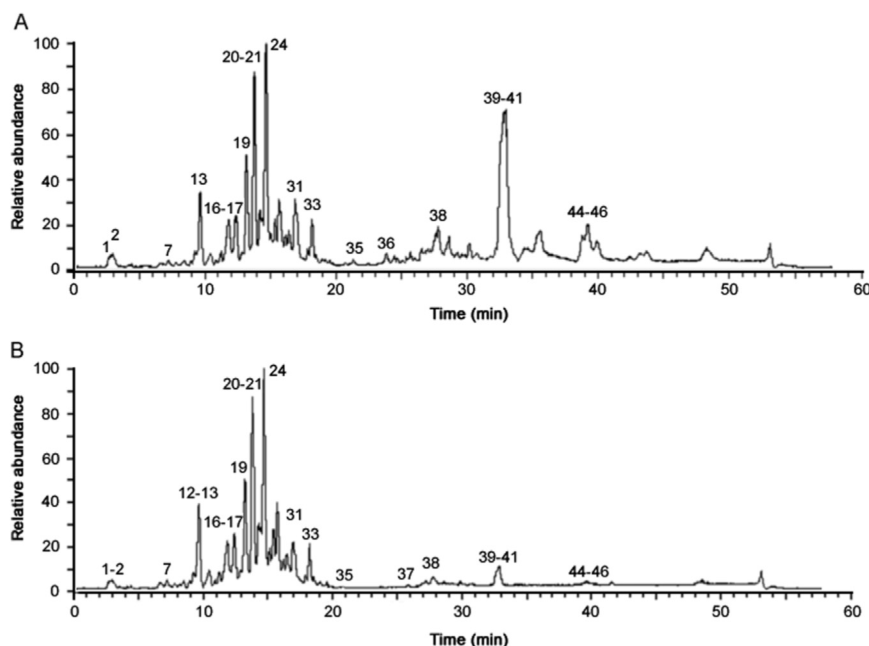

Fig. 2. Chromatographic profile of the tincture A (A) and tincture B (B) of red propolis using LC/ESI/FTMS/Orbitrap.

The same crude extracts were used to prepare the tinctures A and B of the red propolis, which were then injected in LC/ESI/FTMS/Orbitrap (Fig. 2). The confirmation of the presence of isoflavonoids (daidzein, genistein, formononetin, and biochanin A), chalcones (isoliquiritigenin), flavanones (liquiritigenin, pinocembrin and naringenin), pterocarpans (medicarpin), flavones (galangin), and phenolic acids in the red propolis were performed by detecting the ion using the negative mode of the LC/ESI/FTMS/Orbitrap and the formulae were generated by using Xcalibur software from Thermo Fisher Scientific® (Table 2). Other less studied compound classes were also detected in red propolis. In the time period of 24.0–50.0 min, terpenes, propolones and guttiferones were detected in the red propolis. There is a particular interest in guttiferones due to their antibacterial, antileishmanial, anti-HIV and anticancer activities [1,18]. So, guttiferone E was identified as an important compound (MW 602.8) with formulae $C_{38}H_{50}O_6$ at a retention time of 32.9 min during the tinctures A and B analysis.

Using LC/ESI/FTMS/Orbitrap, it was possible to observe similarity of tinctures A and B between the retention time from 2.0 to 22.0 min, which corresponds to the retention time of phenolic acids and isoflavonoids. However, differences were observed in the lowest intensities of the peaks from 24.0 to 50.0 min, mainly for the particular interest peak at 32.9 min (identified as guttiferone E) which was detected in both tinctures. Both HPLC/UV and LC/ESI/FTMS/Orbitrap were useful tools in exploring the chemical characterization of the red propolis extracts. LC/ESI/FTMS/Orbitrap is considered a modern technique for phytochemical screening because it is a universal detector to identify different compounds in trace level with a large range of mass. Furthermore, the LC/ESI/FTMS/Orbitrap is considered a specific detector to identify different markers from propolis, phytochemicals and other apiceuticals, independent of the secondary metabolite classes and it is a choice technique for rapid screening purposes because the Orbitrap mode is used to concentrate ions, keeping the analysis more sensitive and robust while detecting hundreds of compounds in only one analysis. Then, this technique is chosen to analyze chemical profile and to establish a fingerprint of complex samples like functional foods, phytochemicals, apiceuticals and others [1,19].

3.2. *Total flavonoid content*

The chromatograms in Figs. 1 and 2 show a wide range of phenolic compounds present in the red propolis and a great possibility to identify new compounds. The red propolis from the state of Alagoas, Brazil, is an atypical case with regards to the presence of isoflavonoids. It is possible to find many secondary metabolite classes such as flavans (catechin) and isoflavans (vestitol) present in the red propolis without absorption in the visible region and compounds which possess low

Table 2
Identification and confirmation of some markers of the Brazilian red propolis in tinctures using LC/ESI/FTMS/Orbitrap.

Peak	RT (min)	$[M-H]^-$ (m/z)	MW	Formulae	Compound
1	2.95	179.0556	180.16	$C_9H_8O_4$	Caffeic acid
2	2.98	193.0502	194.18	$C_{10}H_{10}O_4$	Ferulic acid
3	3.00	178.0556	179.05	$C_9H_8O_4$	Umbelic acid
4	3.04	163.0243	164.16	$C_9H_8O_3$	p-coumaric acid
5	3.10	475.1232	476.43	$C_{23}H_{24}O_{11}$	7-O-beta-glucopyranosyl-4′-hydroxy-5-methoxyisoflavone
6	4.50	461.1023	462.40	$C_{22}H_{22}O_{11}$	6-Methoxyluteolin 7-rhamnoside
7	7.05	269.0811	270.24	$C_{15}H_{10}O_5$	Genistein
8	7.35	285.0395	286.24	$C_{15}H_{10}O_5$	Kaempferol
9	8.04	289.0711	290.27	$C_{15}H_{14}O_6$	Cathechin
10	8.28	287.0553	288.25	$C_{15}H_{12}O_6$	Dalbergioidin
11	8.83	289.0711	290.27	$C_{15}H_{14}O_6$	Epicatechin
12	8.95	253.0499	254.24	$C_{15}H_{10}O_4$	Daidzein
13	9.70	255.0654	256.27	$C_{15}H_{12}O_4$	Liquiritigenin
14	10.5	283.0384	284.26	$C_{16}H_{12}O_5$	2´-Hydroxyformononetin
15	11.3	331.0810	332.30	$C_{17}H_{16}O_7$	Evernic acid
16–17	11.9	271.0602	272.25	$C_{15}H_{12}O_5$	Narigenin / Pinobanksin
18	12.4	285.0758	286.24	$C_{15}H_{10}O_6$	Calycosin
19	13.4	255.0654	256.27	$C_{15}H_{12}O_4$	Isoliquiritigenin
20–21	13.77	267.0655	268.28	$C_{16}H_{12}O_4$	Formononetin / Isoformononetin
22	14.2	269.0812	270.28	$C_{16}H_{14}O_4$	4,4′-dihydroxy-2-methoxychalcone
23	14.2	269.0812	270.32	$C_{16}H_{14}O_4$	(7 S)-dalbergiphenol
24	14.66	271.0603	272.29	$C_{16}H_{16}O_4$	Vestitol
25	15.10	269.0813	270.28	$C_{16}H_{14}O_4$	Pinostrobin
26	15.10	269.0813	270.27	$C_{16}H_{14}O_4$	Medicarpin
27	16.2	271.0607	272.29	$C_{16}H_{16}O_4$	2′,6′-dihydroxy-4′-methoxydihydrochalcone
28	16.2	283.0657	284.26	$C_{16}H_{12}O_5$	Thevetiaflavone
29	16.42	283.0603	284.26	$C_{16}H_{12}O_5$	Biochanin A
30	16.73	253.0865	254.25	$C_{15}H_{10}O_4$	Chrysin
31	16.87	255.1019	256.27	$C_{15}H_{12}O_4$	Pinocembrin
32	17.0	539.1699	540.56	$C_{32}H_{28}O_8$	3′,4′-di-O-benzyl-7-O-(2-hydroxyethyl)-3-O-methylquercetin
33	18.2	285.1131	286.32	$C_{17}H_{18}O_4$	(3 S)-7-O-methylvestitol
34	18.2	285.1131	286.32	$C_{17}H_{18}O_4$	7,3′-Dihydroxy-4′-methoxy-8-methylflavane
35	21.4	425.1603	426.71	$C_{30}H_{50}O$	Cycloartenol / α-amyrin / β-amyrin
36	23.6	533.2906	534.69	$C_{33}H_{42}O_6$	Hyperibone H
37	25.5	617.3480	618.82	$C_{38}H_{50}O_7$	16-hidroxiguttiferone K
38	27.3	511.1383	512.50	$C_{30}H_{24}O_8$	Rhuschalcone V
39	32.80	601.3533	602.80	$C_{38}H_{50}O_6$	Guttiferone F
40	32.88	601.3533	602.80	$C_{38}H_{50}O_6$	Xantochymol
41	32.90	601.3533	602.80	$C_{38}H_{50}O_6$	Guttiferone E
42	34.10	347.2233	348.52	$C_{22}H_{36}O_3$	Anacardic acid (6-pentadecylsalycilic acid)
43	34.50	509.2744	510.59	$C_{27}H_{38}O_3$	Makassaric acid
44	39.24	669.4355	670.917	$C_{43}H_{58}O_6$	Guttiferone C
45	39.24	669.4355	670.917	$C_{43}H_{58}O_6$	Guttiferone D
46	39.24	669.4355	670.917	$C_{43}H_{58}O_6$	Guttiferone B

RT: Retention time (min), MW: Molecular weight.

reactivity with specific reagents as aluminum chloride in the classical reactions for the total flavonoid determination. Thus, UV spectrophotometry is used for testing the total flavonoid content, which was based on direct reading in a specific wavelength without the presence of chemical chelating reactions and/or derivatization reagents.

In previous studies, tincture, crude extracts, chloroform extract, syrups formulations and microcapsules of red propolis were observed to have maximum absorbance at 280 nm similar to the catechin standard and different from the quercetin standard, which showed maximum wavelengths at 250 nm and 370 nm. Thus, several research groups have developed a rapid method of direct reading in the UV for total flavonoid content [7]. The analytical standard of catechin presents a spectrophotometric (specific) profile similar to the red propolis tinctures and demonstrates greater specificity than quercetin for the total flavonoid content. One of the explanations for the greater specificity of catechin is related to the large amount of isoflavones and isoflavans present in the red propolis tinctures, in which the maximum absorption occurs at 280 nm [7].

The tincture and the catechin standard showed good correlation between the absorbance and the studied concentration range. The tincture presented a straight line equation of y=0.0017833x +0.0133; r^2 =0.9997, while the catechin standard presented a straight line equation of y=0.0011267x +0.005933; r^2 =0.9999, demonstrating that the method can be used to quantify flavonoid/phenolic substances present in extracts, tincture, fractions and microcapsules of the red propolis. The microcapsules loaded with the red propolis extracts presented precision values of 4.30% using freeze-drying, 3.51% using spray-drying and 0.95% for the red propolis tincture in the flavonoid quantitation assays.

Table 3 shows the flavonoid content in the tincture and microcapsules loaded with the red propolis. The B tincture showed that the total flavonoid content corresponds to 21.76 mg of flavonoids/100 mg of the tincture. Microcapsules, using the spray-drying technique, presented higher flavonoid quantities of 29.99–40.79 mg of flavonoids/100 mg of the microcapsules and the microcapsules obtained through freeze-drying presented flavonoid quantities of 20.50–31.61 mg of flavonoids/100 mg of the microcapsules. The spray-drying technique promotes the enrichment of the powder during the drying process due to the loss of low-density excipients or incompatible excipients through the spray-drying process.

Table 3
Total flavonoids content in red propolis tincture and microcapsules using UV–Vis method by direct reading.

Sample	Concentration (μg/mL)	Concentration (mg of flavonoids/ equivalent of 100 mg of tincture in the microcapsule)
Tincture	87.05	21.76
MRPE A-SD	123.17	30.79
MRPE B-SD	163.17	40.79
MRPE C-SD	144.49	36.12
MRPE D-SD	74.97	29.99
MRPE A-FD	82.00	20.50
MRPE B-FD	95.81	23.95
MRPE C-FD	92.87	23.22
MRPE D-FD	126.44	31.61

3.3. Antioxidant activity

The tinctures and microcapsules loaded with the red propolis extract presented good antioxidant activity of 77.12%–98.06% at the concentration of 50 μg/mL (Table 4). The antioxidant activity was also performed at low concentrations (25–2.5 μg/mL) and spray-dried microcapsules presented similar antioxidant activity in comparison to the tinctures A and B of the red propolis and better than the freeze-dried microcapsules. The antioxidant activity and IC_{50} were determined for the tinctures A and B (IC_{50}: 6.95 μg/mL and 7.48 μg/mL, respectively), the spray-dried microcapsules (IC_{50}: 8.89–15.63 μg/mL) the freeze-dried microcapsules (IC_{50}: 11.83–23.36 μg/mL). Antioxidant activities of the red propolis extract and its fractions have been demonstrated between 5.15 and 14.68 μg/mL [1]. The spray-dried microcapsules, obtained through drying thermal process, did not reduce the antioxidant activity of flavonoids, except for MRPE D, which presented a high percentage of pectin in its composition (Table 1). Spray-dried microcapsules might present small cracks or micro-pores on the surface of the wall material of the microcapsules to allow and facilitate the release of flavonoids within the microcapsules. The ice crystals might also be formed during the freeze-drying process, promoting the micelles rupture in the micellar system and resulting in the separation of oil-water system (organic-water) during a long time of drying (24–48 h) of the microcapsules.

3.4. Antibacterial activity and MIC

The susceptibility test, for B tincture and chloroform extracts, displayed activity against the S*taphylococcus aureus* ATCC 25293 and *Pseudomonas aeruginosa* ATCC 27853 in all the concentrations studied (100 and 2000 μg/mL) (Table 5). Júnior et al. [20] reported a lower MIC for chloroform and acetanolic extracts of the Brazilian red propolis in relation to crude ethanol extract (MIC value < 100 μg/mL). Cabral et al. [21] also demonstrated a lower MIC for chloroform extract in relation to the crude ethanol extract of the Brazilian red propolis (MIC value ranging from 16 to 32 μg/mL). In both cases, *S. aureus* was susceptible and used to prove antibacterial activity. Neves et al. [22] found MIC results of 256 μg/mL for crude ethanol extract in both strains of bacteria *S. aureus* and *P. aeruginosa* using the Brazilian red propolis from the state of Pernambuco, Brazil and these antibacterial activities were attributed to the flavonoid formononetin, which presented a MIC result of 200 μg/mL.

The chloroform extract of the red propolis obtained from tincture A and enriched with flavonoids, isoflavonoids and chalcone also showed antibacterial activity in the concentration range of 100–2000 μg/mL for the *S. aureus* and *P. aeruginosa* (Table 5). The lower bacterial activity of the B tincture in relation to the tincture A can be explained due to the extraction process through the maceration of the B extract obtained directly from the crude extract containing a low amount of polyisoprenylated benzophenones (Fig. 2) and a large amount of additional substances without antibacterial activity (greases and waxes). Previous works in literature report antibacterial activity of some guttiferones and propolones against gram-positive and gram-negative strains [23,24], and some authors suggest that the inhibitory activity is due to a synergistic effect between phenolic acids, flavonoids and other organic compounds, especially pinocembrin, pinobanksin and galangin [25], as well as the ability to inhibit cell motility [26]. Xiu and Lee [27] demonstrated the broad-spectrum antibacterial capability of the flavonoid myricetin against gram-positive bacteria (such as MRSA) and gram-negative bacteria (*Klebsiella pneumoniae*) through the inhibition mechanism of protein synthesis. Pepeljnjak and Kosalec [28] demonstrated the synergistic action of ethanol extracts of enriched propolis with phenolic acids and galangin flavonoid against bacteria *Enterococcus spp*., *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA).

Microcapsules loaded with the red propolis extract displayed antibacterial activity in the concentration range of 200–400 μg/mL using agar-diffusion assay. A confirmatory test using the broth microdilution assay confirmed an MIC of 135.87–271.74 μg/mL for the *S. aureus* and 271.74–543.48 μg/mL for the *P. aeruginosa* (Tables 6 and 7). High MIC values were observed for the MRPE D-SD and MRPE B-FD compositions than others, which can explain the formation of insoluble chelanting between excipients and active compounds (flavonoids) in these formulations that occurred during the preparation and drying steps using the spray-drying and freeze-drying techniques. This insoluble chelanting between pharmaceutical excipients and flavonoids promotes a modified release of flavonoids from the microcapsules and results in high MIC values, especially the compositions with a high percentage of guar gum (MRPE B) and a high percentage of pectin

Table 4
Antioxidant activity and IC_{50} of the tinctures and microcapsules loaded with red propolis extract at different concentrations.

Sample	Antioxidant activity (%, mean of three determinations ± standard deviation)					
	50 μg/mL	25 μg/mL	10 μg/mL	5 μg/mL	2.5 μg/mL	IC_{50} (95% CI)
Tincture A	98.06 ± 0.18	89.20 ± 0.30	79.00 ± 0.13	40.73 ± 0.03	25.97 ± 0.04	6.95 (6.27–7.70)
Tincture B	89.65 ± 0.22	88.95 ± 0.23	73.25 ± 0.11	29.29 ± 0.09	16.52 ± 0.10	7.48 (6.86–8.16)
MRPE A-SD	83.29 ± 0.43	83.20 ± 0.40	51.81 ± 0.25	30.82 ± 0.29	15.00 ± 0.29	8.89 (7.67–10.17)
MRPE B-SD	85.77 ± 0.33	81.05 ± 0.55	53.48 ± 0.21	34.54 ± 0.16	19.78 ± 0.16	9.08 (8.24–10.02)
MRPE C-SD	86.07 ± 0.38	84.12 ± 0.39	49.02 ± 0.19	43.17 ± 0.25	23.39 ± 0.35	10.43 (6.73–16.15)
MRPE D-SD	87.86 ± 0.28	70.98 ± 0.65	37.20 ± 0.38	20.84 ± 0.25	11.61 ± 0.35	15.63 (15.01–16.28)
MRPE A-FD	85.38 ± 0.55	65.89 ± 0.36	28.77 ± 0.46	14.38 ± 0.41	9.67 ± 0.35	17.80 (17.76–17.84)
MRPE B-FD	82.78 ± 0.35	72.40 ± 0.45	39.39 ± 0.34	20.75 ± 0.34	8.73 ± 0.35	11.83 (11.14–12.57)
MRPE C-FD	77.12 ± 0.67	53.54 ± 0.55	20.00 ± 0.67	13.44 ± 0.63	5.89 ± 0.45	23.36 (19.88–27.45)
MRPE D-FD	84.69 ± 0.71	81.27 ± 0.42	36.17 ± 0.81	24.80 ± 0.85	10.03 ± 0.65	12.64 (10.66–15.00)

Free radical DPPH sequestering activity (%) of the tinctures of red propolis and different compositions of microcapsules loaded with red propolis extract. IC_{50} (minimal concentration required to obtain 50% antioxidant effect) (95% of confidence interval).

Table 5
Antibacterial activity of tincture B and chloroform extracts A and B of red propolis against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Concentration (μg/mL)	Diameter of inhibition halos (mm)					
	Staphylococcus aureus			*Pseudomonas aeruginosa*		
	Tincture B	Chloroform extract A	Chloroform extract B	Tincture B	Chloroform extract A	Chloroform extract B
2000	14	18	22	28	–	30
1000	12	16	19	28	18	28
800	12	16	21	28	–	26
600	12	14	20	26	18	24
400	10	12	19	24	15	22
200	8	8	17	24	14	18
100	8	8	15	20	12	18

(MRPE D) (Tables 1, 6 and 7). A high percentage of guar gum or pectin promotes a long delay in the release of flavonoids, resulting in high values of MIC for gram-positive (MIC values 271.74–543.48 μg/mL) and gram-negative bacteria strains (MIC values 543.48–1086.96 μg/mL). In general, microcapsules of the red propolis presented an MIC of 135.87–271.74 μg/mL for *S. aureus* and an MIC of 271.74–543.48 μg/mL for *P. aeruginosa*, thus establishing a susceptibility order (*S. aureus* > *P. aeruginosa*), and thus the lower concentrations were capable of inhibiting the growth of *S. aureus* (gram-positive) relative to *P. aeruginosa* (gram-negative) (Tables 6 and 7). These observations have been reported in the literature before [29].

Bruschi et al. [30] showed inhibition zones against *S. aureus* (16.33 ± 0.58 mm) and *P. aeruginosa* (11.00 ± 0.00 mm) against a standardized suspension of bacteria containing 2.5×10^7 CFU/mL using propolis tincture at 30% (m/m), but the spray-dried extracts did not show inhibitory activity. The propolis extract inhibited the oral microorganism growth and the activity of enzymes glucosyltransferases (GTFS) of the *Streptococcus mutans* bacteria. The GTFS enzymes produce α-glucan substances, which are responsible for the adhesion and buildup cariogenic streptococci on the tooth surface and have an essential role in the development of agents related to the formation of dental plaque and caries [31].

The variation in the microbiological results between different research groups can also be explained by factors associated with the extraction technique, different geographical origin (flora diversity), the season in which the resin was collected and the presence of any contaminants [32]. The microbiological methods, culture medium and microbiological bioburden can influence the results obtained. Eloff et al. [33] demonstrated the sensitivity of the broth microdilution method in the MIC determination, which was 32 times higher than the disk-diffusion technique and therefore the broth microdilution method is considered the gold standard method [34].

The assays of antibacterial activity and MIC test, through the disk-diffusion method and the broth microdilution method, proved to be an excellent tool for the microbiological quality control of the extracts, tinctures and microcapsules of the red propolis. Moreover, the microbiological methods show high sensitivity in differentiating extracts, tinctures and microcapsules from different techniques and are used as a tool in a batch-to-batch quality control of bulk products and apiceuticals.

4. Conclusion

HPLC/UV and LC/ESI/FTMS/Orbitrap identified different secondary metabolite classes such as isoflavones, chalcones, pterocarpans, flavonones, flavones, phenolic acids, terpenes and guttiferones in the Brazilian red propolis tinctures. LC/ESI/FTMS/Orbitrap was a useful tool in the confirmation of different chemical markers of the red propolis, and demonstrated the complexity of this apiceutical product.

The tincture and microcapsules of the red propolis presented high flavonoid quantities specially for the spray-dried microcapsules. The tinctures and spray-dried microcapsules presented similar antioxidant activity and were better than the freeze-dried microcapsules.

The tinctures and microcapsules proved to be bioactive against gram-positive and gram-negative bacteria; moreover, the gram-positive bacteria were more sensible than the gram-negative bacteria. The LC/ESI/FTMS/Orbitrap and microbiological methods were sensitive and could distinguish the quality of the tinctures and the microcapsule compositions. Thus, the tinctures and microcapsules of the red propolis have a potential application for nutraceutical products.

Table 6
Antibacterial activity and MIC of the different MRPE obtained through spray-drying and freeze-drying against *S. aureus*.

Concentration (μg/mL)	Diameter of inhibition halos (mm)							
	Spray-drying				Freeze-drying			
	MRPE A-SD	MRPE B-SD	MRPE C-SD	MRPE D-SD	MRPE A-FD	MRPE B-FD	MRPE C-FD	MRPE D-FD
2000	16	16	16	14	16	18	16	16
1500	16	14	16	12	14	16	14	16
1000	16	14	14	12	14	16	14	14
800	14	14	14	12	14	14	14	14
400	12	12	12	10	12	12	12	12
200	10	10	10	8	10	8	10	10
100	8	8	8	8	8	8	8	8
50	8	8	8	8	8	8	8	8
MIC (μg/mL)	135.87–271.74	135.87–271.74	135.87–271.74	271.74–543.48	135.87–271.74	271.74–543.48	135.87–271.74	135.87–271.74

Table 7
Antibacterial activity and MIC of the different MRPE obtained through spray-drying and freeze-drying against *P. aeruginosa*.

Concentration (μg/mL)	Diameter of inhibition halos (mm)							
	Spray-drying				Freeze-drying			
	MRPE A-SD	MRPE B-SD	MRPE C-SD	MRPE D-SD	MRPE A-FD	MRPE B-FD	MRPE C-FD	MRPE D-FD
2000	20	22	20	16	20	16	18	20
1500	18	20	18	14	18	14	16	18
1000	16	16	16	12	16	10	16	16
800	14	16	14	10	14	10	14	14
400	12	14	12	8	12	8	12	12
200	10	12	10	8	10	8	10	10
100	8	8	8	8	8	8	8	8
50	8	8	8	8	8	8	8	8
MIC (μg/mL)	271.74–543.48	271.74–543.48	271.74–543.48	543.48–1086.96	271.74–543.48	543.48–1086.96	271.74–543.48	271.74–543.48

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to acknowledge CNPq (The Brazilian National Council for Scientific and Technological Development), CAPES (The Brazilian Coordination for the Personal Improvement of Superior Education) and FAPEAL (Foundation for Sponsoring Research in the State of Alagoas) for the scholarships of the Master's course in Nutrition (PPGNUT) and CNPq and FINEP (The Brazilian Financer for Studies and Projects) for financial support (Grant number 478390/2010-6) according to the financial aid to the researchers 14/2010-Universal/MCT/CNPq and 14/2014-Universal/MCT/CNPq. The authors would also like to thank the Microbiological Quality Control Laboratory for Food analysis of the Nutrition College of the Federal University of Alagoas and to the Beekeepers: José Marinho de Lima (*in memorian*) and José Izaias Zacarias dos Santos for their support in collecting raw material and for the red propolis donations.

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