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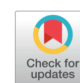
RESEARCH PAPER

Brazilian red propolis extracts: study of chemical composition by ESI-MS/MS (ESI+) and cytotoxic profiles against colon cancer cell lines



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Abstract Red propolis is a natural resin mixture produced by honeybees and presents a source of active compounds with a variety of biological activities. In this study, we describe the chemical characterization and potential antitumor activity of total extract of Brazilian red propolis and its fractions. Fractions were obtained through column chromatography revealing 14 different compounds in all samples, which were determined and distinguished of other isobar molecules by fragmentation pathways by ESI-MS/MS in positive mode. Some molecules as cis-asarone or trans-isoelemicin were identified and distinguish from elemicin compound and vestitol or isovestitol were also distinguished from neovestitol by fragmention pathway. Other important compounds as liquiritigenin was differentiated from isoliquiritigenin and formononetin from dalbergin.

MTT viability assay showed different toxicity in cell lines after exposition to total extract and fractions. Fractions 05 and 06 had more selectivity against HT-29 and HCT-116 cancer cells, respectively, in relation to normal cells. IC_{50} (ranging of 72.45 ± 6.57 to 73.58 ± 1.00 µg/mL) in cancer cells were lower than reported in total extracts of propolis. May-Grunwald/Giemsa staining revealed cellular morphological changes after exposition to higher concentrations of red propolis extracts. Fractionation techniques can contribute to reduce chemical diversity verified in propolis mixtures, generating fractions with improved biological activity and contributing to the development of new strategies for discovery of natural compounds against cancer.

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Introduction

Propolis is a bee resin used by centuries in traditional medicine to curative proposes (Kuropatnicki, Szliszka, & Krol, 2013). The word *propolis* is originated from Greek words *pro* (''in front of'', ''in defense of'') and *polis* (''community'') and define a substance used by bees to seal and protect beehives against invaders and pathogenic microorganisms (Toreti, Sato, Pastore, & Park, 2013).

Honeybees, of *Apis mellifera* (L.) specie, produce propolis through the collection of resins from leaves, buds and barks of certain tree species (Catchpole, Mitchell, Bloor, Davis, & Suddes, 2015), with addition of wax and salivary enzimes which promote hydrolization of phenolic compounds, improving the pharmacological activities (Najafi, Vahedy, Seyyedin, Jomehzadeh, & Bozary, 2007).

The chemical composition of propolis is strongly related to the geographic location, botanical sources and bee species (Huang, Zhang, Wang, Li, & Hu, 2014; Rufatto et al., 2017). Propolis is composed of 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% other substances such as minerals and organic molecules (phenolic acids, esters, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes and β-esteroids) (Silva-Carvalho, Baltazar, & Almeida-Aguiar, 2015). More than 300 compounds have been identified in different samples and new ones are still being recognized by analytical methods. Chemical profile can be also modified by seasonality and climatic conditions, which makes even more complex the chemical identification of samples (Wagh, 2013).

Several types of propolis are reported in the world, each one presenting different biological activities and chemical profiles (Bankova, 2005). Red propolis has been found in several countries such as Cuba, Mexico and China. In Brazil, occurs 13 types of propolis and the red one, recently described, occurs at northeastern region (states of Alagoas, Bahia, Paraiba, Sergipe and Pernambuco) in mangrove biomes (López, Schmidt, Eberlin, & Sawaya, 2014; Silva et al., 2008).

The botanical origin of Brazilian red propolis has been attributed to the specie *Dalbergia ecastophyllum* (L.) Taub. due to the chemical correspondence found in propolis samples and resins produced by this plant (Daugsch, Moraes, Fort, & Park, 2008). Natural compounds found in propolis have shown important pharmacological effects such as antimicrobial, antioxidant, cytotoxic, anti-inflammatory and anti-allergic activities (Kamiya, Nishihara, Hara, & Adachi, 2012; Lopez et al., 2015). In relation to chemical composition, reports using red propolis have indicated a great diversity of molecules such as elemicin, formononetin, liquiritigenin, isoliquiritigenin, biochanin A, medicarpin, homopterocarpan, quercetin and vestitol, that can differentiate red propolis of other Brazilian types (Mendonça et al., 2015).

Studies have reported potent activity of red propolis against cancer cells, decreasing cellular migration and inducing apoptosis (Begnini et al., 2014). Enriched fractions with xanthochymol and formononetin can promote cell death by activation of a caspase-3-dependent pathway (Novak et al., 2014). Hep-2 cells (human laryngeal epidermoid carcinoma) exposes to red propolis fractions have shown apoptotic events and DNA fragmentation (Frozza et al., 2017). Liquiritigenin, compound also present in propolis, have shown suppression of tumor growth by reduction of angiogenesis in murine model (Liu et al., 2012). Osteosarcoma cells treated with biochanin A showed cell death by cleavage of PARP, a capase-3-activation dependent pathway and formation of apoptotic bodies (Hsu et al., 2018), indicating that compounds of propolis could be promising for anticancer treatments.

Cancer is a major public health problem worldwide (Siegel, Miller, & Jemal, 2016). In Brazil, colon cancer is the third most prevalent neoplasia diagnosed in men and second in women and has been associated with hereditary and lifestyle factors such as alimentary habits with excessive consumption of red meat and processed foods, a small consumption of fruits and sedentarism (INCA, 2016).

Given the wide pharmacological activity, including possible anticancer effects and the complex composition found in total extracts of propolis, recent studies have focused their work in fractionating methods with objective to produce enriched fractions, with lower chemical diversity and improved biological effects. However, until present, few studies have evaluated the effects of these fractions against tumor cell lines and no reports have verified the activity of Brazilian red propolis fractions against colon cancer cells. In this context, the aim of this work was to investigate the activity of different fractions obtained from Brazilian red propolis, identifying and elucidating possible compounds and active fractions against colorectal cancer cells.

Materials and methods

Red propolis sample and preparation of total extract

Red propolis sample was collected in 2013 from state of Alagoas (Northeast region of Brazil), stored at room temperature and ground to a fine powder with liquid nitrogen. Firstly, to remove waxes and resins, 50 g of propolis were sonicated with 150 mL of hexane (30 min). After, the soluble compounds were removed by filtration and the remaining solid were sonicated again with solvent renovation. This procedure was repeated three times. Ultrasound-assisted extractions were carried out using 500 W Sonics Vibracell equipment at working amplitude of 60% at 0 °C. The remaining solid from previous procedure was used for extraction with ethyl acetate (150 mL × 3 times), using the same extraction, time and filtration conditions. The ethyl acetate extracts were combined and subsequently evaporated to dryness using a rotary evaporator (40 °C) followed by lyophilization. The ethyl acetate total extract (EAE) was stored at −20 °C until utilization.

Fractionation

The fractionation was conducted using a column chromatography (4 × 45 cm) with 147 g of silica gel and 4 g of EAE. Hexane (100–60%) and ethyl acetate (0–40%) was used as mobile phase. 255 tubes were collected and analyzed by thin layer chromatography (TLC) using mobile phase constituted by hexane:ethyl acetate (7:3). The TLC plates

were visualized under UV light at 254 and 365 nm and stained with vanillin-sulphuric acid spray, followed by heating. The collected tubes with similar chromatographic profiles were grouped and the solvent was removed by rotary evaporator (40 °C) followed by lyophilization. From these steps, 11 fractions were obtained and kept at −20 °C.

Chemical characterization

EAE and fractions were diluted in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid. The solutions were infused directly or with HPLC (Shymadzu) assistance into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate 10 μL.min^{-1}. ESI(+)–MS were acquired using a hybrid high-resolution and high accuracy microTOF-QII mass spectrometer (Bruker® Daltonics) under the following conditions: capillary and cone voltages were set to +3500 V and +40 V, respectively, with a de-solvation temperature of 100 °C. Diagnostic ions were identified by the comparison of exact *m*/*z* with compounds determined in previous studies (Table 1). For data acquisition and processing, Hystar software (Bruker® Daltonics) was used. The data were collected in the *m*/*z* range of 70–800 at the speed of two scans per second.

Cell culture and cytotoxicity assay

HT-29 (human colorectal adenocarcinoma) and HCT-116 (human colorectal carcinoma) cancer cell lines and Vero (monkey kidney epithelial) non-tumor cell line were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. HT-29 and HCT-116 cells were grown in RPMI 1640 medium and Vero cells were cultivated with Dulbecco's Modified Eagle's Medium High-Glucose (DMEM), both supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and 1% (v/v) penicillin–streptomycin. Cells lines were kept at 37 °C in a humidified atmosphere with 5% of CO_2 and 95% air. The study was performed at 70–80% of cell confluence.

EAE and fractions were submitted to sequential dilutions with DMSO (0.5%) and culture medium. Cell viability was measured using the MTT assay (Mosmann, 1983). Initially, cells (1×10^4 cells/well) were seeded into 96-well plates with 100 μL of supplemented culture medium for 24 h for attachment. After, cells were treated with EAE and fractions in a range of concentrations from 5 to 150 μg/mL, for 24 h. Negative controls were treated only with DMSO (0.5%) at culture medium. Then, the treatment was removed and a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (1 mg.mL^{-1}) was added and incubated for two hours. MTT solution was removed and the formazan crystals were dissolved with DMSO for 30 min. Absorbance was measured using a microplate reader (Spectra Max 190, Molecular Devices) at 570 nm. The IC_{50} (concentration that inhibit 50% of cell viability) was calculated in relation to control cells (100% of viability). Each experiment was performed in triplicate and repeated at least three times.

Morphological assay

Vero, HT-29 and HCT-116 cells were seeded in 24 well plates (2×10^4 cells/well) with supplemented media (DMEM or RPMI) with 10% of bovine fetal serum and 1% of penicillin-streptomicin. After 24 h, the culture media was removed and the cells were treated with EAE and fractions at concentrations of 35 μg/mL and 70 μg/mL, for 24 h. Vehicle (solution of DMSO 0.5% in culture medium) was used as control. The cells were stained with May-Grunwald/Giemsa for 3 min in the same plate. After washing, cells were observed in microscope with 20× magnification.

Statistical analysis

The results for MTT were expressed as means ± standard deviation obtained from three independent experiments. Statistical significance was evaluated using t-test and one-way analysis of variance (ANOVA) with *post hoc* multiple comparisons procedure (Tukey test) to assess statistical differences in normal distribution. A *p*-value <0.05 was considered significant using the Statistical Package for Social Sciences (SPSS, version 19.0) for Windows.

Results

Chemical composition

In this study, values of cut-off were defined to select active or inactivate fractions. Fractions with IC_{50} higher than 150 μg/mL (fraction 01 to 03) were considered inactive and were excluded from the next steps. The chemical composition of EAE and active fractions (04 to 11) was determined with HPLC/MS and ESI-MS/MS (ESI+) in a Q-TOF (micrOTOF-QII Bruker Daltonics) with UFLC.

Chemical analysis revealed 14 compounds in EAE and fractions (Fig. 1 and Table 1), which are determined and distinguished of other isobar molecules by fragmentation pathways by ESI-MS/MS in positive mode. Full spectra of EAE and fractions are available at Supporting Information.

The compounds were identified based on the information provided by HMRS as exact *m*/*z* and fragmentation pathway in accordance with the literature. The acceptable experimental error to confirm chemical compounds was established as 5 ppm, which provides highly secure identification of the chemical compounds.

Viability assay

Cytotoxic activity of the EAE and fractions were investigated through MTT assay. The treatment with EAE did not reveal significant statistical differences among normal (Vero) and cancer cell lines (HT-29 and HCT-116) (Table 2).

The fractions more active in this study were fractions 05 and 06, with lower effects in normal cells and showing activity against HT-29 and HCT-116, respectively. By this reason, these fractions were selected for the morphological assay.

Table 1 Chemical characterization of ethyl acetate total extract (EAE) from red propolis and their fractions (fractions 04 to 11).

Entry	Sample	Identification	Elem. Comp. (+H)	Precursor ion *m/z*	Diff. ppm	Fragmentation ions (%) [MS-MS]	Reference
1	EAE	Methoxyeugenol	$C_{11}H_{15}O_3$	195.1017	2.15	–	Alencar et al. (2007)
2	EAE	cis-asarone (a) or trans-isoelemicin (b)	$C_{12}H_{17}O_3$	209.1168	4.64	209.1164 (58); 194.0987 (100); 168.0827 (85) 166.0531 (23).	Righi et al. (2011), Trusheva et al. (2006)
3	EAE; 04	(2S)-7-hydroxyflavanone	$C_{15}H_{13}O_3$	241.0877	4.69	241.0909 (27); 137.0273 (100); 131.0529 (50); 103.0566 (10)	Awale et al. (2008)
4	EAE	Chrysin	$C_{15}H_{11}O_4$	255.0650	2.88	255.0697 (100); 151.0436 (98); 131.0598 (12)	López et al. (2014)
5	EAE; 08; 09; 10; 11	Liquiritigenin	$C_{15}H_{13}O_4$	257.0820	2.40	257.0854 (33); 239.0774 (7); 229.0889 (16); 211.0792 (9); 147.0481 (49); 137.0273 (100) 133.0689 (10); 119.0556 (9)	Daugsch et al. (2008), Frozza et al. (2013)
6	EAE; 07; 08; 09; 10	Formononetin	$C_{16}H_{13}O_4$	269.0861	4.30	269.0851 (100); 254.0617 (61); 237.0567 (34); 226.0679 (84); 213.0961 (52); 197.0638 (39); 137.0268 (14); 118.0440 (26)	Awale et al. (2008)
7	EAE; 04; 05; 06; 11	Medicarpin	$C_{16}H_{15}O_4$	271.0981	3.93	271.1023 (30); 161.0645 (9); 137.0631 (100); 123.0467 (8)	Alencar et al. (2007), Frozza et al. (2013)
8	EAE; 07; 08; 09	Vestitol(a) or Isovestitol(b)	$C_{16}H_{17}O_4$	273.1116	3.97	273.1152 (41); 163.0804 (12); 149.0636 (27); 137.0634 (100); 123.0478 (95)	Awale et al. (2008), Piccinelli et al. (2011)
9	EAE; 05; 06; 07; 08; 09; 10; 11	Biochanin A	$C_{16}H_{13}O_5$	285.0772	3.16	285.0808 (100); 270.0585 (79); 253.0584 (22); 242.0637 (71); 229.0900 (46); 213.0613 (26); 170.0260 (34); 152.0113 (24); 137.0631 (52)	Awale et al. (2008)
10	EAE; 04	Homopterocarpin	$C_{17}H_{17}O_4$	285.1128	0.41	285.1129 (72); 270.0928 (13); 257.1297 (22); 177.0603 (44); 163.0770 (16); 149.0628 (15); 137.0628 (100)	Piccinelli et al. (2011)
11	EAE; 07	(3S)-Vestitone	$C_{16}H_{15}O_5$	287.0920	1.92	287.0977 (100); 269.0798 (24); 241.0972 (41); 167.0379 (53); 163.0443 (8); 153.0552 (22); 137.0631 (73)	Awale et al. (2008)

Table 1 (*Continued*)

Entry	Sample	Identification	Elem. Comp. (+H)	Precursor ion *m/z*	Diff. ppm	Fragmentation ions (%) [MS-MS]	Reference
12	EAE; 04; 05; 06	7-O-Methylvestitol	$C_{17}H_{19}O_4$	287.1295	4.06	287.1185 (21); 163.0794 (16); 137.0628 (100).	Piccinelli et al. (2011)
13	EAE; 04	(3S)-violanone	$C_{17}H_{17}O_6$	317.1037	3.74	371.1130 (54); 299.1045 (22); 289.1074 (28); 271.0968 (15); 165.0558 (100); 151.0487 (18); 137.0590 (26)	Awale et al. (2008)
14	EAE; 06; 07; 08	Guttiferone E or Xan-thochymol	$C_{38}H_{51}O_6$	603.3673	2.10	603.3733 (100); 467.2452 (51); 411.1865 (72); 343.1225 (63); 137.1331 (15)	Trusheva et al. (2006), Novak et al. (2014)

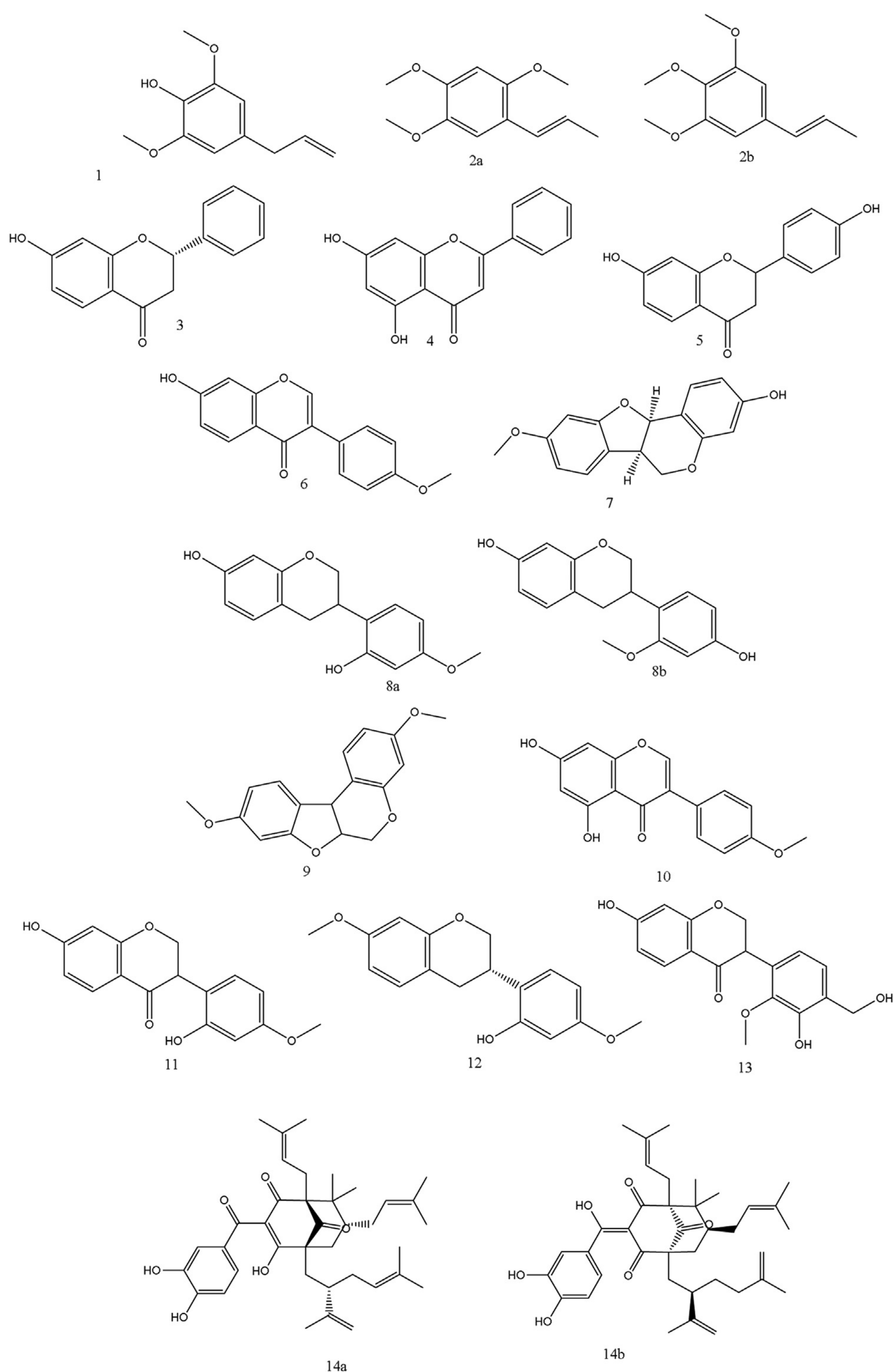

Figure 1 Structure of compounds identified in the red propolis ethyl acetate total extract (EAE) and fractions. The entry number according to Table 1 identifies each structure.

Although fraction 11 showed higher level of cytotoxic activity in all cell models in relation to the other fractions and EAE no significant statistical differences between normal and cancer cells were observed. These results suggest that selectivity and chemical composition are related to each fraction and could produce different levels of cytotoxicity.

Morphological assays

EAE and fractions 05 and 06 were tested against normal and cancer cell lines using concentrations of 35 and 70 μg/mL (1/2 IC_{50} and IC_{50} concentrations) to evidence possible morphological alterations. These fractions were selected given its enhanced activity against HT-29 and HCT-116 cell lines,

Table 2 IC50 in normal and cancer cell lines after exposition to ethyl acetate total extract (EAE) from red propolis and their fractions.

Treatments	IC_{50}		
	Vero	HT-29	HCT-116
EAE	68.52 ± 4.72 a	75.15 ± 3.35 a	70.81 ± 4.18 a
Fraction 04	81.68 ± 6.18 a	68.33 ± 5.97 b	90.38 ± 5.63 a
Fraction 05	103.25 ± 5.35 a	73.58 ± 1.00 b	83.42 ± 4.41 a
Fraction 06	107.61 ± 6.91 a	93.80 ± 7.56 b	72.45 ± 6.57 b
Fraction 07	78.35 ± 6.93 a	69.04 ± 7.56 a	70.83 ± 4.02 a
Fraction 08	92.96 ± 6.00 a	77.67 ± 6.76 b	83.86 ± 2.26 a
Fraction 09	95.37 ± 9.21 a	105.23 ± 3.61 b	82.71 ± 3.96 a
Fraction 10	98.25 ± 4.70 a	81.14 ± 2.02 b	99.17 ± 8.10 a
Fraction 11	40.32 ± 5.64 a	49.88 ± 6.83 a	41.68 ± 3.25 a

Results of IC_{50} expressed as mean ± standard deviation of three independent replicates. Different letters in the same line indicate statistical difference in relation to control cell (Vero) ($p < 0.05$, ANOVA One-way).

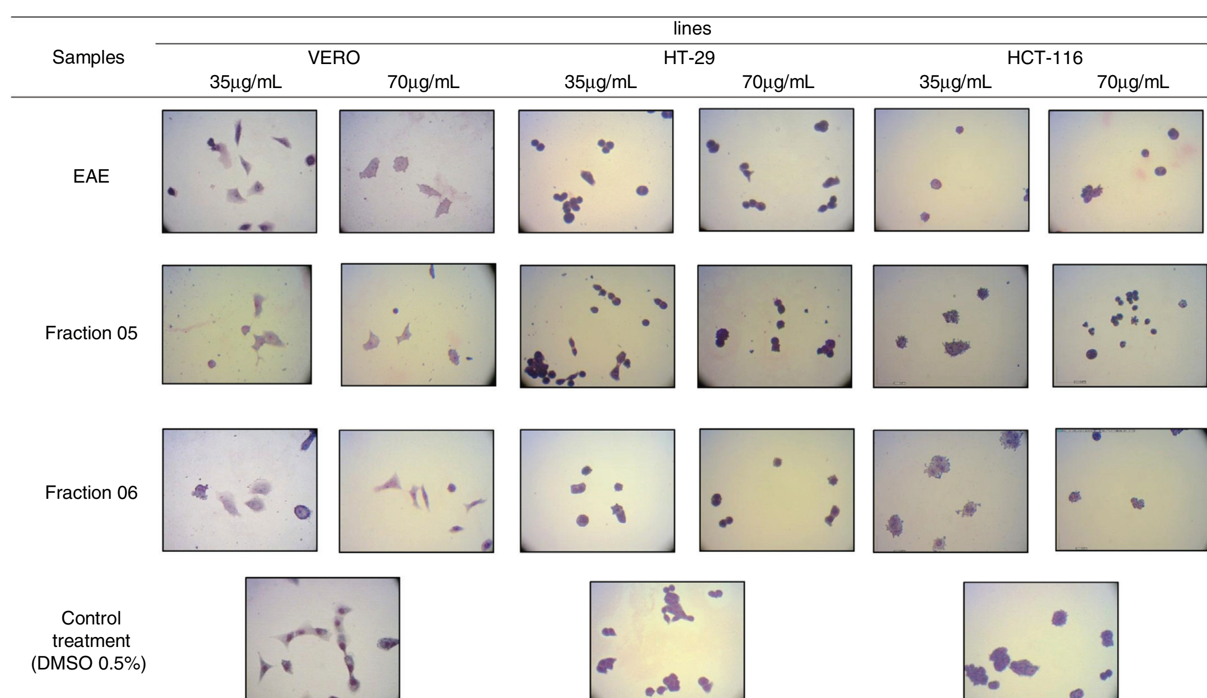

Figure 2 Morphology of normal and cancer cell lines exposed to red propolis fractions, analyzed by May-Grunwald staining.

with lower effects in Vero cells. Evidences of apoptotic alterations were observed such as cell shrinkage, detachment and cytoplasm retraction (Fig. 2). It was observed that cells exposed to higher concentrations of fractions (70 µg/mL) resulted in increased morphological alterations in relation to lower concentrations (35 µg/mL). These data also confirm the results for IC_{50} obtained by MTT assay (Table 2).

Discussion

Propolis has been used since ancient times and has gained attention from the scientific community as a potential source of natural drugs given its biological activities. The mixture of compounds found in this natural resin is largely dependent of the geographical origin and botanical sources (Bankova, Popova, & Trusheva, 2014), presenting a very complex composition constituted mainly of waxes, essential oils and phenolic compounds (Awale et al., 2008).

Several studies have applied crude extracts in cytotoxic investigations, in special for anticancer screening (Freires, de Alencar, & Rosalen, 2016; Watanabe, Amarante, Conti, & Sforcin, 2011). However, the large chemical diversity present in the total extracts may mask the real pharmacological effects. Also, some of the active compounds may be in low concentrations to provide relevant biological activity and chemical identification by analytical methods could be difficult to perform (Bankova, 2005; Harvey, Edrada-Ebel, & Quinn, 2015).

Identification of chemical profile of natural products requires high precision methods such HPLC and ESI-MS/MS

to determine the chemical diversity present in extracts and fractions originated from natural sources (Mendonça et al., 2015; Nunes & Guerreiro, 2012).

Our study has identified 14 compounds in total extract and fractions. By HPLC and ESI-MS/MS techniques were possible to identify and distinguish the presence of cis-asarone or trans-isoelemicin from elemicin compound (m/z 209.1168), by fragment 194.0987 that indicate the loss of a methyl group [-CH_3].

Vestitol or isovestitol, m/z 273.1116, were also identified from neovestitol by fragmention pathway, where the formation of ions m/z 149.0636 and 123.0478 are not possible at neovestitol compound by fragmentation process. Liquiritigenin was differentiated of isoliquiritigenin (m/z 257.0820) by formation of ion m/z 137.0273. In a similar way, the fragmentation data have indicated the presence of formononetin (m/z 269.0861) instead dalbergin (m/z 269.0861) by presence of ion 137.0268. In both cases, occurred loss of carbon monoxide [−CO] from parental ions, making possible the correct identification of these compounds. The data obtained by ESI-MS/MS corroborate with other investigations conducted by our lab (Frozza et al., 2013; Frozza et al., 2017) and other researches that elucidate the structures of propolis compounds (Awale et al., 2008; Li, Awale, Tezuka, & Kadota, 2008).

Formononetin, identified in the fractions, is classified as one of chemical markers of red propolis. This compound is found in Brazilian and Cuban extracts (Cuesta-Rubio et al., 2007; Frozza et al., 2017; Mendonça et al., 2015) promoting tumor regression in mice with prostate cancer (Li et al., 2014). Another study, using red propolis fraction, has identified formononetin and xanthochymol as main compounds, with growth inhibition on the development of cancer cells through a caspase-3-dependent pathway (Novak et al., 2014). In colorectal cancer, formononetin was efficient to induce apoptosis on *in vivo* and *in vitro* models (Huang et al., 2015), showing promising perspectives for clinical applications (Wu et al., 2015).

The flavonoids liquiritigenin, biochanin A and medicarpin identified in this work were cited in previous studies of our group using a total extract (Frozza et al., 2013). Liquiritigenin had shown *in vivo* cancer inhibition mediated by cellular effects and inhibition of angiogenesis (Liu et al., 2012). Vestitol, also found in the fractions, showed anti-inflammatory properties by inhibitory activity in neutrophil migration, suggesting modulation of the immune system (Franchin et al., 2016).

Phenolic compounds originated from plant or synthetic sources have shown interesting anti-proliferative effects in breast, cervical, colon, leukemia, lung, prostate and skin tumor cell lines (Roleira et al., 2015). Moreover, polyphenolic content is directly linked to antioxidant activities (Mouhoubi-Tafinine, Ouchemoukh, & Tamendjari, 2016) and to several health benefits such as prevention of major diseases (Boudet, 2007).

Propolis also shows a great potential in biotechnology research. Biocellulose membranes enriched with propolis have improved wound healing and tissue regeneration in murine models (Barud et al., 2013). Natural rubber latex membranes loaded with red propolis have showed potent antimicrobial activity and no toxic effects were verified in fibroblast cells, showing promising results for skin treatments (Zancanela et al., 2018). Chitosan-based nanoparticles incorporated with propolis had showed cytotoxic effects in HepG2 cells (hepatocellular carcinoma) with apoptosis induction and reduction of proliferative G0/G1 phase, indicating that propolis compounds could be used in new strategies for anticancer treatments (Elbaz, Khalil, Abd-Rabou, & El-Sherbiny, 2016).

In this study, different levels of cytotoxicity were found after exposition of normal and cancer cells against EAE and propolis fractions. EAE, fraction 07 and fraction 11 did not shown differences of IC_{50} between cancer and normal cells lines.

Fractions 05 and 06 presented better results of cytotoxicity against cancer cells, HT-29 and HCT-116, respectively, with lower effects on normal cells. Biochanin A found in fraction 05, also showed anti-proliferative effects in HT-29 cells, with apoptotic induction and improving effects of radiotherapy (Puthli, Tiwari, & Mishra, 2013). This molecule also has showed activity in other cancer cells, inducing cell death and cell cycle arrest with effects in several cancer types as prostate cancer, breast, hepatocellular carcionoma and tumors of central nervous system, opening new perspectives to development of combined treatments using this molecule to cancer treatment (Raheja, Girdhar, Lather, & Pandita, 2018). Another interesting compound, 7-O-MethylVestitol, found in fraction 05, also showed potent inhibitory effects against HT-1080 and Colon 26-LS cells of colon adenocarcinoma (Li, Awale, Tezuka, & Kadota, 2008).

Fraction 06 presents a compound identified as guttiferone E or xanthochymol, which was absent in the fraction 05. These isomers have been considered as an inseparable mixture and has been identified in lipophilic extracts of Brazilian red propolis (Fasolo, Bergold, von Poser, & Teixeira, 2016). Guttiferone E induces endoplasmic reticulum response that produces growth inhibition on colon cancer cell lines such as HCT-116, HT-29 and SW480 (Protiva et al., 2008). This compound can induce apoptosis *via* activation of caspases 8 and 9, effector caspase 3/7 and loss of mitochondrial membrane potential (Kuete et al., 2013). A study using xanthochymol revealed the induction of cell cycle arrest and apoptosis in cell models (Novak et al., 2014).

These results could indicate that the existence of selectivity or synergism effects of propolis compounds. Polyphenols can act improving activities in biological models, where some compounds can act activating important targets or cell pathways or, in the other hand, protecting the cells against deleterious effects (Choi et al., 2013). Flavonoids can induce antioxidant effects in dose-dependent manner, protecting cells and tissues of free radicals, but flavonoids with pro-oxidant effects also could be used as anticancer drugs, opening new possibilities of treatments (Lahouel et al., 2007).

Fractions 08 and 10, also efficient against HT-29, showed liquiritigenin in the chemical analysis. This compound can act as an antiproliferative molecule, inhibiting migration and invasion processes in cells B16F10, as well as *in vivo* models. This compound is also associated to apoptosis induction on HeLa cells by release of cytochrome c (Liu et al., 2011).

IC_{50} results, observed on selective fractions 05 and 06 against tumor cells, were lower than observed in other studies using total extracts. Ethanolic extracts of propolis

have revealed activity on human bladder cancer cells (IC_{50} of 95 μg/mL) (Begnini et al., 2014) and HeLa (81.40 μg/mL) cells (Frozza et al., 2013). Similar results to our study were found with fraction L of red propolis in Hep-2 cells lines (IC_{50} of 74.60 μg/mL) (Frozza et al., 2017), indicating that fractionation techniques can improve the biological effects of propolis.

Morphological cell changes varied according to propolis concentration exposure with more alterations in cellular morphology at higher concentrations. Data from literature also indicate cell alterations mediated by ethanolic extracts of propolis in tumor cell lines, showing detachment, apoptotic blebbing, reduction in size and density at higher exposures (Choudhari, Haghniaz, Rajwade, & Paknikar, 2013). Biochanin A found in the fractions also produce morphological alterations in colon cancer cells, contributing to apoptosis and cell death (Puthli et al., 2013).

The results here presented indicate that chemical compounds found in fractions and EAE were able to produce *in vitro* cytotoxic effects and the fractionation is an efficient way to produce samples with lower chemical diversity and better biological activities. Also, this study can confirm that compounds from Brazilian red propolis can induce different effects in tumor cells. Thus, the correct identification of compounds and active fractions and their effects in cell lines can improve new studies aiming the application of natural drugs in biological systems, that can be used with biotechnology tools, improving the development of new treatments and strategies for anticancer therapy.

Conclusion

Researches involving natural products can bring new target compounds and strategies for treatment of human diseases such as colon cancer. Our study has indicated that Brazilian red propolis, especially after fractionation, can produce several cytotoxic activities against cancer cells. Bioguided fractionations are efficient methods to improve the development of new drugs, evidencing the activity of each group of compounds, individually or in combination with others.

The methodology used in this study was efficient to produce fractions with different chemical composition that could be applied to discover new compounds from propolis and contribute to the understanding of isolated activity of each compound, improving the knowledge and favoring the development of target therapeutics against colorectal cancer.

Conflicts of interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biori.2019.02.001.

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