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Brazilian Green Propolis: Chemical Composition of Essential Oil and Their In Vitro Antioxidant, Antibacterial and Antiproliferative Activities

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HIGHLIGHTS

- The chemical composition of propolis varies according to the region where it is produced.
- Production of green propolis in Minas Gerais state, Brazil, is related to the high amount of resin yielded by *Baccharis dracunculifolia.*
- Major compounds found in essential oil from Brazilian green propolis were carvacrol, acetophenone, spathulenol, (*E*)-nerolidol and β-caryophyllene.
- Brazilian green propolis showed high antibacterial, antioxidant and antiproliferative activities.

Abstract: Propolis is a resinous substance collected and processed by *Apis mellifera* from parts of plants, buds and exudates. In Minas Gerais (MG) state, Brazil, green propolis is produced from the collection of resinous substance found in shoot apices of *Baccharis dracunculifolia*. This paper aims to investigate the chemical composition and in vitro antioxidant, anti-*Helicobacter pylori*, antimycobacterial and antiproliferative activities of essential oil (EO) from Brazilian green propolis (BGP-EO). The oil showed high antibacterial activity against *H. pylori* (MIC = 6.25 µg/mL), *Mycobacterium avium* (MIC = 62.5 µg/mL) and *M. tuberculosis* (MIC = 64 µg/mL). Its antioxidant activity was evaluated in vitro by both DPPH (IC_{50} = 23.48 µg/mL) and ABTS (IC_{50} = 32.18 µg/mL) methods. The antiproliferative activity in normal (GM07492A, lung fibroblasts)

and tumor cell lines (MCF-7, HeLa and M059J) was analyzed by the XTT assay. BGP-EO showed inhibition of normal cell growth at 68.93 ± 2.56 µg/mL. Antiproliferative activity was observed against human tumor cell lines, whose IC_{50} values were 56.17, 66.43 and -65.83 µg/mL for MCF-7, HeLa and M059J cells, respectively. Its major constituents, which were determined by GC-FID and GC-MS, were carvacrol (20.7 %), acetophenone (13.5 %), spathulenol (11.0 %), (*E*)-nerolidol (9.7 %) and β-caryophyllene (6.2 %). These results showed the effectiveness of BGP-EO as a natural product which has promising biological activities.

Keywords: free radicals; *Helicobacter pylori*; *Mycobacterium tuberculosis*; *Mycobacterium avium*; tumor cell lines; *Baccharis dracunculifoli*

INTRODUCTION

Propolis is a resinous material which is used by honeybees (*Apis mellifera* L.) in hive construction in order to seal exterior wall cracks and prevent insects, such as cicadas, butterflies, moths and beetles, from invading their hives. Propolis is also used inside beehives, since it protects bees against pathogenic microorganisms, such as bacteria, fungi and viruses [1].

Since bees produce it from substances secreted by different plant species, specificities of native vegetation are responsible for the variability found in its chemical composition worldwide [2]. *Baccharis dracunculifolia*, whose common names in Portuguese are '*vassourinha*', '*alecrim do campo*' and '*alecrim de vassoura*', is native to Brazil and the biological precursor of green propolis. It belongs to the family Asteraceae and has been known for yielding phenolic compounds and essential oils (EO) that bestow high antiulcer, antibacterial and antifungal activities [3-5].

Propolis is a natural product which has promising antioxidant potential since it acts as a body defense agent against free radicals that are found in all organisms. Its composition includes chemical constituents that protect organisms against chronic diseases caused by oxidative stress, such as cancer and metabolic disorders [6].

Some studies have not only shown that propolis exhibits satisfactory antibacterial activity against *Helicobacter pylori*, but have also highlighted its anti-inflammatory and anesthetic activities [7]. Besides, flavonoids, terpenoids, simple phenolics, pterocarpans, phenylethanoid derivatives, stilbenes and lignans are classes of compounds – found in propolis – which are responsible for its anti-*Mycobacterium tuberculosis* activity, a fact that justifies its use in folk medicine to treat tuberculosis, for instance [8].

Antiproliferative activity of propolis has also drawn researchers' interest worldwide, since studies have proven that it may be applied as a nutritional supplement during cancer treatments. In addition, several reports have shown antiproliferative effects of propolis from different origins and their fractions in several cancer cell lines [9-10].

Taking into account the pharmacological potential of extracts and EOs extracted from samples of propolis found all over the world [11-12] and, mainly, from samples of EOs and compounds found in Brazilian green propolis [13-16], this study aimed at determining the chemical composition and in vitro antioxidant, anti-*Helicobacter pylori*, antimycobacterial and antiproliferative activities of EO from Brazilian green propolis (BGP-EO) found in São Lourenço, a city located in the south of Minas Gerais (MG) state, Brazil.

MATERIAL AND METHODS

EO extraction and GC-FID and GC–MS analyses

Fresh green propolis (500 g) produced by *A. mellifera* L. (*B. dracunculifolia*) in the Atlantic Forest in São Lourenço (22º 06' 59" S and 45º 03' 16" W), MG, Brazil, was purchased at Apiário Esperança (São Lourenço, MG, Brazil) on June 15th, 2017. EO from Brazilian green propolis (BGP-EO) was extracted by hydrodistillation with the use of a Clevenger-type apparatus for 3 h. Hydrodistillation was performed in quintuplicate. To this end, the material was divided into five 100 g samples and 500 mL distilled water was added to each sample. After manual collection of BGP-EO, traces of water which remained in the oil were removed with anhydrous sodium sulfate; filtration followed. BGP-EO was stored in an amber bottle and kept in a refrigerator at 4 °C until analysis. EO yield was calculated from green propolis and expressed as the average of quintuplicate analyses.

Gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC–MS) analyses were performed by Shimadzu QP2010 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. GC-MS and GC-FID conditions and BGP-EO identification have been previously reported [17]. Identification

of volatile components of BGP-EO was based on their Kovats retention index on an Rtx-5MS capillary column under the same operating conditions used for GC, relative to a homologous series of *n*-alkanes (C_8-C_{20}). Structures were computer-matched with the Wiley 7, NIST 08 and FFNSC 1.2 spectral libraries while their fragmentation patterns were compared with literature data [18].

Antioxidant assay

Free radical scavenging activities of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and azino-bis (ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$) were determined by a spectrophotometric method [19], with modifications. In the DPPH assay, different concentrations of BGP-EO in methanol (10–100 µg/mL) were added to 2 mL 0.1 mM solution of DPPH that was previously prepared and incubated in the dark for 30 min. Absorbance was recorded at 517 nm by a UV spectrophotometer. In the ABTS assay, 1980 mL diluted $ABTS^{+}$ solution was added to 20 µL BGP-EO, previously diluted in ethanol. Absorbance at 734 nm was measured 6 min after initial mixing. BHT was used as positive control. Assays were carried out in triplicate. Inhibition percentage was calculated as (I%) = (A0 − A/A0) × 100, where A0 is the absorbance of the control and A is the absorbance of the samples. IC_{50} value was calculated as the concentration of sample required to scavenge 50% of free radicals by graphing the I% versus EO concentration.

Anti-*Helicobacter pylori* assay

Minimum inhibitory concentration (MIC in µg/mL) of BGP-EO was calculated by the broth microdilution method on 96-well microplates. The following ATCC reference strain was used: *Helicobacter pylori* (ATCC 43526). Evaluation of the activity of EO with reference drugs was made by comparing bacterial growth on each plate of *H. pylori*. BGP-EO was dissolved in 5% dimethyl sulfoxide to reach final concentrations ranging between 0.195 and 400 µg/mL. The inoculum was adjusted at 625 nm in a spectrophotometer to produce a cell concentration equal to 5 x 10^5 CFU/mL. Plates were incubated in a CO_2 incubator at 37 °C for 3 days under microaerobic conditions. Tetracycline, at concentrations ranging from 0.115 to 59.0 µg/mL, was employed as the standard drug and incubated under the previously mentioned conditions. After incubation, 30 µL 0.01% aqueous resazurin solution was added to each well to evaluated microbial growth [20]. In addition, plates were incubated at 35 °C for 72 h under microaerophilic conditions and the MBC (Minimum Bactericidal Concentration) was defined as the lowest concentration of BGP-EO without any growth of microorganisms. MBC/MIC ratio was calculated to determine either bactericidal or bacteriostatic effects of BGP-EO under study.

Antimycobacterial assay

Mycobacteria *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and *Mycobacterium avium* (ATCC 25291) were obtained from the American Type Collection (ATCC) and maintained at – 80 °C. Antimycobacterial activity of BGP-EO was evaluated by the MIC broth microdilution method conducted on microplates. Resazurin was employed to reveal mycobacterial growth by the Resazurin Microtiter Assay (REMA) method [21], with modifications. BGP-EO was diluted (two-fold) with Middlebrook 7H9 broth (DifcoTM, Detroit, MI, USA). The *Mycobacterium* inoculum was then added to BGP-EO solutions to obtain concentrations ranging from 50 to 2000 µg/mL. Afterwards, inoculated plates were incubated at 37 °C for 42 days (1st reading was carried out after 28 days while the 2nd one occurred after 42 days) and inhibition percentage was determined [22]. Isoniazid was used as positive control at concentrations (MIC =1.47 µg/mL) whereas Middlebrook 7H9 broth and the inoculum were used as solvent and negative control, respectively.

Antiproliferative assay

In this study, three different tumor cell lines were used: human breast adenocarcinoma (MCF-7), human cervical adenocarcinoma (HeLa) and human glioblastoma (M059J). A normal human cell line (lung fibroblasts, GM07492A) was included to evaluate possible selective activity of the natural product under investigation. Different cell lines were maintained as monolayers in plastic culture medium (HAM-F10 + DMEM, 1:1, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich) and 2.38 mg/mL Hepes (Sigma-Aldrich). Cells were incubated at 36.5 °C in humidified 5% CO_2 atmosphere. Antiproliferative activity was measured by the in vitro Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics), in agreement with the manufacturer's instructions. In the experiments, cells (10^4 cells/well) were incubated on 96-well microplates. Each well was filled with 100 µL HAM-F10/DMEM medium which contained EO at concentrations ranging from 3.91 to 500

µg/mL. Negative (no treatment) solvent (0.02% DMSO, dimethylsulfoxide, Sigma-Aldrich) and positive (doxorubicin, DXR, Pharmacia Brasil Ltda.) controls were included. After incubation at 36.5°C for 24 h, the culture medium was removed. Cells were washed with 100 µL PBS (phosphate buffered saline) to remove treatments and exposed to 100 µL culture medium HAM-F10 without phenol red. Then, 25 µL XTT was added and cells were incubated at 36.5°C for 17 h. Sample absorbance was determined by a multi-plate reader (ELISA – Tecan – SW Magellan vs 5.03 STD 2P) at wavelength of 450 nm and reference length of 620 nm. Antiproliferative activity was evaluated with the use of IC_{50}, the concentration capable of inhibiting 50% of cell line growth as a response parameter, which was calculated by the GraphPad Prism program that plotted cell survival against concentrations of the natural product under investigation. One-way ANOVA was used for comparing means ($p < 0.05$). Experiments were performed in triplicate. The selectivity index was calculated by dividing the IC_{50} value of the BGP-EO obtained for GM07492A cells by the IC_{50} value obtained for the cancer cell line.

RESULTS AND DISCUSSION

Yield of EO extracted from green propolis, collected in Brazil, was 0.30 ± 0.15% (w/w). Major compounds found in BGP-EO were carvacrol (20.7 %), acetophenone (13.5 %), spathulenol (11.0%), (*E*)-nerolidol (9.7%) and β-caryophyllene (6.2%) (Table 1).

Previous reports of BGP-EO only showed terpenes β-caryophyllene (13.4%), (*E*)-nerolidol (17.1%) and selina-3,7(11)-diene (10.4%) as its major constituents [23]. Fernandes-Silva and coauthors studied EO from green propolis found in Viçosa, MG, Brazil, and reported the following major constituents: 3-prenylcinnamic acid allyl ester (26.3%), spathulenol (23.4%) and 7-phenyl-5-oxo-heptanol (13.3%) [14]. Taking into consideration that *B. dracunculifolia*, which is the botanical source of Brazilian green propolis, has been the most studied and exported type of propolis [24], it should be highlighted that, among the previously mentioned compounds, only spathulenol, β-caryophyllene and (*E*)-nerolidol were identified as major constituents of BGP-EO. Carvacrol, the most abundant chemical constituent in BGP-EO had also been previously isolated from *B. dracunculifolia* leaves [25].

Chemical constituents identified in BGP-EO may be directly related to the chemical composition of essential oils from *B. dracunculifolia*, since bees collect raw material from this plant species to yield this type of propolis. Mainly regarding this species of Asteraceae, EO from *B. dracunculifolia* collected in Pitangui, MG, exhibited high concentration of nerolidol (30.62%) [5]. On the other hand, when it was collected in Viçosa, MG [26], its EO was very similar to BGP-EO, even though most BGP-EO major constituents were found at lower concentrations.

Thus, the origin of green propolis produced by *A. mellifera* in MG, Brazil, should be highlighted. It is a resinous substance collected in the shoot apices of *B. dracunculifolia*. It has a complex chemical composition, whose main components are EO, phenols, polyphenols, flavanones, chalcones and prenyilates derived from *p*-coumaric acid [4]. It is also relevant to mention that bees collect regardless of gender (male or female) and phonological state (flowering or vegetative); the period of high resin collecting visits coincides with the harvest of green propolis in MG [4].

Table 1. Chemical composition of BGP-EO

Compounds	**%RA**	**RI_{exp}**	**RI_{lit}**
Benzaldehyde	0.3	969	970
Limonene	0.4	1030	1031
Acetophenone	**13.5**	1076	1078
Perillene	1.1	1097	1099
Linalool	0.9	1106	1107
Benzyl nitrile	0.8	1147	1148
3-Ethyl-phenol	1.6	1171	1171
α-Terpineol	1.5	1188	1189
Decanal	0.3	1206	1207
Geraniol	0.2	1227	1228
Geranial	0.4	1272	1270
Thymol	3.0	1288	1289
Carvacrol	**20.7**	1297	1298
Citronellyl acetate	0.6	1354	1354
a-Copaene	3.9	1370	1372
β-Elemene	1.9	1374	1375
α-Gurjunene	1.9	1408	1410
β-Caryophyllene	**6.2**	1418	1418
Aromandendrene	5.2	1446	1447
Humulene	2.3	1455	1455
Alloaromadendrene	2.3	1460	1460
γ-Muurolene	0.2	1475	1477
Viridiflorene	0.9	1503	1505
γ-Cadinene	1.0	1512	1513
δ-Cadinene	1.8	1524	1524
Cubenene	1.2	1533	1533
Selina-3,7(11)-diene	1.0	1547	1547
(*E*)-Nerolidol	**9.7**	1562	1564
Spathulenol	**11.0**	1578	1578
Viridiflorol	1.6	1592	1593
α-Cadinol	0.3	1651	1652
Total	**97.7**		

RI_{exp}: Retention index relative to *n*-alkanes (C_8-C_{20}) on the Rtx-5MS (30 m X 0.25 mm; 0.250 µm) column; **RI_{lit}:** Retention index found in the literature [18]; **%RA:** relative area (peak area relative to the total peak area in the GC-FID chromatogram).

Regarding antioxidant activity, the results show that BGP-EO exhibited significant DPPH free radical activity, with IC_{50} values of 23.48 ± 10.11 µg/mL. In the azino-bis (ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) method, the value of IC_{50} = 32.18 µg/mL was a little higher. The positive control was BHT (butylated hydroxytoluene), whose IC_{50} = 19.66 ± 0.07 µg/mL. BHT was chosen to be the positive control because it inhibits both free radical formation and lipid peroxidation effectively [27]. The high antioxidant activity exhibited by BGP-EO may be explained by its major constituents, i. e., the ones that have already had their antioxidant activity reported by the literature: acetophenone [28], carvacrol [29], (*E*)-nerolidol [30], β-caryophyllene [31] and spathulenol [32]. Green propolis has been highlighted as a natural product that exhibits antioxidant activity, which has recently been evaluated by both DPPH e $ABTS^+$ methods for the extract [33].

Antibacterial activity of BGP-EO was evaluated against the *H. pylori* strain; its MIC was measured by the broth microdilution method. BGP-EO proved highly active against *H. pylori* with MIC = 6.25 µg/mL. The positive control was tetracycline whose MIC = 1.0 µg/mL. BGP-EO has shown promising antibacterial activity against *H. pylori* (MIC = 6.25 µg/mL and MBC = 12.5 µg/mL – MBC/MIC = 2). According to Malm and coauthors, MBC/MIC values of samples ≤ 4 suggested their bactericidal activity [34]. It should be highlighted that antibacterial agents are usually regarded as bactericidal if MBC/MIC is up to four-fold the MIC [35]. Anti-*Helicobacter pylori* activity of green propolis extract has already been described by the literature [36]. The chemical and biological study of BGP-EO reported by this paper adds new and valuable information to the promising antibacterial activity of this type of propolis.

Antibacterial activity of BGP-EO was evaluated against the *M. tuberculosis* and *M. avium* strains; its MICs were measured by the broth microdilution method. BGP-EO proved active against *M. tuberculosis* and *M. avium* with MICs = 64 µg/mL and 62.5 µg/mL, respectively. The positive control was isoniazid whose MIC = 1.47 µg/mL. Some authors have considered that MICs ≤ 200 µg/mL of extracts and EO indicate good activity against *M. tuberculosis* [37]. In addition, according to Holetz and coauthors natural products with MIC values lower than 100 µg/mL, between 100 and 500 µg/mL, from above 500 to 1000 µg/mL, and greater than 1000 µg/mL display good antibacterial activity, moderate antibacterial activity, weak antibacterial activity, and absence of antibacterial activity, respectively [38]. Interestingly, propolis has been used as an ingredient in traditional cures for tuberculosis. Previous in vitro studies have shown that extracts from propolis can inhibit the growth of *M. tuberculosis* and synergise the effect of established antitubercular drugs, such as isoniazid, rifampicin and streptomycin [8]. The excellent antibacterial activity against *M. tuberculosis*, *M. avium* and *H. pylori* may be related to the concentrations of its major constituents, whose bactericidal potential has already been proven. They are carvacrol [39], (*E*)-nerolidol [30], spathulenol [40], β-caryophyllene [41] and acetophenone [42].

Regarding antiproliferative activity, BGP-EO cytotoxicity was evaluated against the GM07492A normal cell line, whose IC_{50} was 68.93 ± 2.56 µg/mL, and against MCF-7, HeLa and M059J tumor cell lines whose IC_{50} values were 56.17 ± 8.41, 66.43 ± 0.40 and 65.83 ± 0.79 µg/mL, respectively (Table 2). The positive control was doxorubicin and IC_{50} values are also shown in Table 2. The lowest IC_{50} value was observed for MCF-7 cells, whose SI was 1.22. Even though the activity of EO against tumor cell lines has not been totally clarified yet, Gautam and coauthors stated that mechanisms underlying antiproliferative activity of EO and constituents may reach several ways of cell cycle regulation, which may often overlap [43]. They include the ones involved in apoptosis, cell growth interruption, antimetastatic and antiangiogenic activities, besides the one that leads to increase in the yield of reactive species, such as oxygen. Taking into account that EO are complex mixtures of several bioactive constituents, its synergic activity should also be considered, since synergism may be responsible for their large number of biological activities [44]. However, cytotoxicity of carvacrol, the major constituent of BGP-EO, has been observed against cancer cells [45]. Studies have shown that carvacrol induced cell death mediated apoptosis [46, 47].

Table 2. Concentration inhibiting 50% growth (IC_{50}) and selectivity index (SI) of BGP-EO against different cell lines

Cell line	Treatment (µg/mL)		
	BGP-EO		DXR
	IC_{50}	SI	IC_{50}
GM07492A	68.93 ± 2.56	-	0.50 ± 0.20
MCF-7	56.17 ± 8.41	1.22	62.10 ± 2.00
HeLa	66.43 ± 0.40	1.03	5.30 ± 1.30
M059J	65.83 ± 0.79	1.04	16.20 ± 2.50

Doxorubicin (DXR) was used as positive control. GM07492A, human lung fibroblasts; MCF-7, human breast adenocarcinoma; HeLa, human cervical adenocarcinoma; M059J, human glioblastoma. The selectivity index is the ratio between the IC_{50} value of BGP-EO obtained for GM07492A cells and the value found for the tumor cell line. Values are mean ± SD, n = 3.

CONCLUSION

Since several classifications of Brazilian propolis, such as yellow, brown, red and green ones, exhibit important biological activities, they have drawn researchers' interest worldwide. Results of this study showed the promising activities of green propolis collected in São Lourenço, a city located in the south of MG, Brazil. Thus, there was evidence that BGP-EO may constitute an alternative source of compounds with promising activities, such as antioxidant, anti-*Helicobacter pylori*, antimycobacterial and antiproliferative ones. In sum, these data are valuable, since they show the potential application of BGP-EO to the development of new drugs. The literature has shown that compounds that had previously been identified in EO from *B. dracunculifolia* and green propolis can guarantee the authenticity of the plant material, green propolis resin and their products. Therefore, these studies corroborate the use of this plant to produce green propolis by honeybees. However, further in vivo studies are needed to ensure and elucidate the mechanism of action of this natural product.

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