Revista Brasileira de Farmacognosia 29 (2019) 807–810

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Short communication

Biological properties of volatile oil from Brazilian brown propolis

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ARTICLE INFO

Article history:
Received 7 May 2019
Accepted 27 July 2019
Available online 5 September 2019

Keywords:
Apis mellifera
Resin
Balsamic substances
Volatiles
Apitherapy

ABSTRACT

Propolis, is a bee product collected from exudates and flower buds of several plants, has strong aroma and several biological applications. This study aimed at evaluating the chemical composition and *in vitro* antioxidant, antibacterial and cytotoxic properties of volatile oil from Brazilian brown propolis. It was extracted by hydrodistillation and analyzed by gas chromatography-flame ionization detection and gas chromatography-mass spectrometry. Volatile oil from brown propolis exhibited strong antibacterial activity against *H. pylori* (MIC 3.25 μg/ml), *Mycobacterium tuberculosis* (MIC 50 μg/ml) and *M. avium* (MIC 62.5 μg/ml). It was evaluated *in vitro* for antioxidant activity by DPPH (IC_{50} 25.0 μg/ml) and ABTS (IC_{50} 30.1 μg/ml) methods. Its cytotoxic property was evaluated in normal (human fibroblasts, GM07429A) and tumor (MCF-7-human breast adenocarcinoma; HeLa-human cervical adenocarcinoma and M059J-human glioblastoma) cell lines. IC_{50} values were 81.32 μg/ml for GM07429A and 85.00, 129.40 and 84.12 μg/ml for MCF-7, HeLa and M059J cells, respectively. Three major dereplicated components of volatile oil from brown propolis were acetophenone (15.2%), nerolidol (13.3%), and spathulenol (11.6%). Our results contribute to a better understanding of the chemical and biological properties of Brazilian brown propolis and provide evidence for its potential medicinal use.

Introduction

Propolis is a resinous substance that bees produce by gathering material from different types of plants. They use it as a sealant to protect their hives from invasive organisms. Major constituents of propolis include phenolic compounds which are represented by flavonoids, phenolic acids and their esters; they attribute promising biological activities, such as antimicrobial, anti-inflammatory and antioxidant ones, to propolis. Even though the chemical composition of propolis may vary, it usually comprises 50% of resin and plant balm, 30% of wax, 10% of volatile oils, 5% of pollen and 5% of other substances, such as organic residues (Wagh, 2013).

Wide application of propolis to modern medicine has drawn increasing attention to its chemical composition. Its composition includes chemical constituents that protect organisms against chronic diseases caused by oxidative stress, such as cancer and metabolic disorders. Thus, this natural product has promising antioxidant potential since it acts as a body defense agent against free radicals that are found in all organisms (Calegari et al., 2017). In addition, previous *in vitro* studies have demonstrated that extracts of propolis could inhibit the growth of *Mycobacterium tuberculosis* as well as synergise the effect of established antitubercular drugs such as isoniazid (Ali et al., 2018).

The cytotoxic 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. Several reports have shown cytotoxic effects of propolis from different

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https://doi.org/10.1016/j.bjp.2019.07.004

origins and their fractions in several cancer cell lines (Pratsinis et al., 2010; Borges et al., 2011).

Literature has reported advances in the use of propolis and its components, mainly regarding its promising and specific anti-*Helicobacter pylori* activity. Some studies show that gastric cancer is strongly related to infection caused by the *Helicobacter pylori*; it leads to an inflammatory process, with consequent induction of oxidative stress, and to pre-neoplastic conditions (Boyanova et al., 2005). It should be emphasized that several biological activities described by the literature refer to extracts from propolis, rather than from its volatile oils. As a result, it should also be highlighted that this study is the first report of antioxidant, anti-*Helicobacter pylori*, antimycobacterial and cytotoxic properties of volatile oil extracted from a type of Brazilian brown propolis.

Considering the well-known pharmacological potential of volatile oil from Brazilian brown propolis (Fernandes et al., 2015), this study aimed to evaluate the chemical composition and *in vitro* antioxidant, antibacterial and cytotoxic properties of samples collected in Borda da Mata, in the south of Minas Gerais (MG) state, Brazil.

Material and methods

Fresh brown propolis (500 g) produced by *Apis mellifera* L. in the Atlantic Forest region in Borda da Mata, MG, was purchased from Apiário Karina (Borda da Mata, MG, Brazil) on June 15th, 2017.

Volatile oil from brown propolis (EOP) was obtained by hydrodistillation for 3 h in a Clevenger-type apparatus and stored at 4 °C up to GC–MS, GC-FID and bioassays. Gas chromatography-flame ionization detection and gas chromatography–mass spectrometry analyses were performed by Shimadzu QP2010 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. GC–MS and GC-FID conditions and the identification of chemical constituents of EOP were carried out in agreement with the methodology proposed by Lemes et al. (2018).

Free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and azino-bis (ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$) were determined by a spectrophotometric method (Justus et al., 2018), with modifications. In the DPPH assay, different concentrations of EOP 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 EOP previously diluted in ethanol and 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 $(I\%) = (A0 - A/A0) \times 100$, 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 volatile oil concentration.

Minimum inhibitory concentration (MIC in μg/ml) of EOP was calculated by the broth microdilution method on 96-well microplates. The following ATCC standard strain was used: *Helicobacter pylori* (ATCC 43526). An evaluation of the activity of the volatile oil with reference drugs was made by comparing bacterial growth on each plate of *H. pylori*. EOP 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 cell concentration equal to 5×10^5 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, incubated under the same conditions previously mentioned.

Table 1
Chemical composition of volatile oil from brown propolis collected in the Atlantic Forest biome, in southeastern Brazil.

Compounds	RI_{exp}	RI_{lit}	%RA
Benzaldehyde	962	961	0.4
β-Pinene	980	981	0.1
Limonene	1035	1036	0.2
Acetophenone	1077	1078	15.2
trans-Linalool oxide	1087	1088	0.4
2-Methoxy-phenol	1089	1090	0.1
Linalool	1097	1098	2.0
Ho-trienol	1109	1110	0.1
Benzyl nitrile	1142	1143	0.6
α-Terpineol	1182	1183	1.4
Thymol	1287	1288	0.2
Carvacrol	1299	1299	0.5
Bicycloelemene	1315	1316	0.2
Eugenol	1347	1348	0.2
α-Copaene	1365	1367	4.5
β-Elemene	1380	1381	0.9
α-Yalangene	1383	1383	0.2
α-Gurjinene	1400	1401	2.1
β-Caryophyllene	1416	1418	5.9
Aromadendrene	1435	1436	4.5
α-Humullene	1450	1451	2.7
allo-Aromadedrene	1454	1455	2.6
γ-Muurolene	1476	1477	1.5
β-Selinene	1483	1484	1.1
Ledene	1490	1490	3.4
δ-Cadinene	1511	1513	5.2
α-Cadinene	1523	1524	2.4
Cadina-1.4-diene	1531	1533	2.9
Nerolidol	1565	1566	13.3
Spathulenol	1582	1582	11.6
Viridiflorol	1592	1593	1.0
Caryophyllene oxide	1611	1613	1.0
α-Muurolol	1642	1643	2.9
α-Cadinol	1656	1656	2.2
Total			93.5

RI_{exp}: Retention index relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; RI_{lit}: Retention index. %RA: relative area.

After incubation, 30 μl 0.01% aqueous resazurin solution was added to each well in order to evaluated microbial growth.

Mycobacteria *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and *M. avium* (ATCC 25291) were obtained from the American Type Collection (ATCC) and maintained at −80 °C. Antimycobacterial activity of EOP was evaluated by the MIC broth microdilution method conducted on 96-well microplates. The methodology used for evaluating antimycobacterial activity of EOP was the one described by Melo et al. (2017).

In this study, three different human tumor cell lines were used: breast adenocarcinoma (MCF-7), cervical adenocarcinoma (HeLa) and glioblastoma (M059J) (Table 3). A normal human cell line (fibroblasts, GM07492A) was included to evaluate whether EOP has selective activity. The methodology used for evaluating cytotoxic activity of EOP was the one described by Silva et al. (2019).

Results and discussion

Brown propolis collected in southeastern Brazil, yielded high content of volatile oil (0.8%) by hydrodistillation. To identify the constituents of EOP, GC-FID and GC-MS analyses were performed and Table 1 shows the 34 constituents with relative amounts above 0.1%, which corresponds to 93.5% of identified constituents. Acetophenone (15.2%), nerolidol (13.3%), β-caryophyllene (5.9%), spathulenol (11.6%) and δ-cadinene (5.2%) were the major components of EOP. Only nerolidol and spathulenol were found in high amounts in EOP in Mato Grosso do Sul state, Brazil; acetophenone was not identified (Fernandes et al., 2015). On the other hand, only three major constituents were identified in EOP found in Piauí,

Table 2
In vitro antioxidant and antibacterial activities of volatile oil from brown propolis (IC_{50} and MIC).

	IC_{50} (μg/ml)	Bacteria	MIC (μg/ml)
DPPH	25.0 ± 10.20	*H. pylori* (ATCC 43526)[a]	3.25
ABTS	30.1 ± 8.11	*M. avium* (ATCC 25291)[b]	62.5
BHT	19.3 ± 0.09	*M. tuberculosis* (ATCC 27294)[b]	50

Results are expressed as mean ± SD, n = 3. IC_{50}: concentration of drug in μg/ml that caused 50% of DPPH• and $ABTS^+$ inhibition. BHT: Positive control.
[a] Positive control used: tetracycline (MIC = 1.0 μg/ml).
[b] Positive control used: isoniazid (MIC = 1.47 μg/ml).

Table 3
Cytotoxic activity (IC_{50} values) of volatile oil from brown propolis against different human cell lines.

Cell line	Treatment (μg/ml)	
	Volatile oil from brown propolis IC_{50}	DXR IC_{50}
GM07492A	81.32 ± 2.48	0.50 ± 0.20
MCF-7	85.00 ± 2.67	62.10 ± 2.00
HeLa	129.40 ± 4.90	5.30 ± 1.30
M059J	84.12 ± 5.39	16.20 ± 2.50

Doxorubicin (DXR) was used as positive control. GM07492A, human lung fibroblasts; MCF-7, human breast adenocarcinoma; HeLa, human cervical adenocarcinoma; M059 J, human glioblastoma. Values are mean ± SD, n = 3.

in northeastern Brazil: β-caryophyllene, caryophyllene oxide and viridiflorol (Sousa et al., 2006).

Concentrations of volatile compounds were found to be quite different from the ones found in the literature. It may be explained by the high variability found in plant species that grow in regions close to beehives, since bees collect plant exudates. Propolis composition depends on seasonality, illumination, altitude, collector type, food availability and the activity developed during propolis exploration. These factors influence the chemical composition of oil extracted all over the world directly; as a result, these differences are believed to contribute significantly to chemical properties and beneficial effects of all types of propolis (Toreti et al., 2013). Specifically, propolis explored from the hive may contain a mixture of resins from various plant sources and beeswax. If individual sources of resin are needed for chemical analysis, it may be necessary to collect the resin from plant tissue or from the hindlegs of returning resin foragers (Bankova et al., 2016).

Regarding antioxidant activity, results show that EOP exhibited significant DPPH• free radical activity, with IC_{50} values of 25.0 μg/ml. When tested by the azino-bis (ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) method, the value of IC_{50} = 30.1 μg/ml was a little higher. The positive control was BHT (butylated hydroxytoluene), whose IC_{50} = 19.3 μg/ml (Table 2).

Antioxidant activity of EOP determined by the DPPH• (2,2-diphenyl-1-picrylhydrazyl) and azino-bis (ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) methods were considered significant by comparison with the positive control BHT (butylated hydroxytoluene), whose IC_{50} was 19.3 μg/ml. BHT was chosen to be the positive control because it inhibits both free radical formation and lipid peroxidation effectively (Bajpai et al., 2017). The good antioxidant activity exhibited by EOP may be explained by its major constituents, mainly the sesquiterpene spathulenol (Nascimento et al., 2018).

Antibacterial activity of EOP was evaluated against *H. pylori*, *M. tuberculosis* and *M. avium* strains; its MIC values were measured by the broth microdilution method. EOP proved highly active against *H. pylori*, *M. tuberculosis* and *M. avium* with MIC = 3.25 μg/ml, 50 μg/ml and 62.5 μg/ml, respectively (Table 2). Positive controls were tetracycline (MIC = 1.0 μg/ml) for anti-*Helicobacter pylori* activity and isoniazid (MIC = 1.47 μg/ml) for antimycobacterial one. This result is relevant, since previous results reinforced that volatile oils whose MIC = 250 μg/ml were considered moderately active against *H. pylori* (Kirmizibekmez et al., 2017). EOP had a greater against *Helicobacter pylori* activity than the volatile oil from *Apium nodiflorum* leaves, whose MIC = 12.5 μg/ml enabled it to be defined as having appreciable value (Menghini et al., 2010). EOP has also shown promising antimycobacterial activity against *Mycobacterium tuberculosis* (MIC = 50 μg/ml) and *M. avium* (MIC = 62.5 μg/ml). According to Holetz et al. (2002), natural products with MIC values below 100 μg/ml, between 100 and 500 μg/ml, from 500 to 1000 μg/ml and above 1000 μg/ml exhibit good, moderate, weak and absence of antibacterial activity, respectively.

The promising antibacterial activity exhibited by EOP may be explained by synergic or even antagonistic effects among its different compounds. Most constituents of volatile oils belong to the family terpenoids; most of them have apolar character and may easily permeate the phospholipid bilayer of biological membranes. These constituents usually exhibit antimicrobial activity because they are capable of breaking through the lipid structure, cause damage to the integrity of the membrane, unbalance intracellular pH and, consequently, lead to cell death (Raut and Karuppayil, 2014).

EOP cytotoxicity was assessed against the GM07492A normal cell line, whose IC_{50} was 81.32 μg/ml and against MCF-7, HeLa and M059 J tumor cell lines whose IC_{50} were 85.00, 129.40 and 84.12 μg/ml, respectively (Table 3). The positive control was doxorubicin and IC_{50} values are also shown in Table 3.

Cytotoxic activity of EOP was observed against normal human and tumor cell lines employed by this study, but without selectivity. Specifically, the main mechanisms that mediate cytotoxic effects of volatile oils include the induction of cell death by activation of apoptosis and/or necrosis processes, cell cycle arrest and loss of function of volatile organelles (Raut and Karuppayil, 2014). It is important to highlight that the cytotoxicity assay also showed that EOP exhibits antibacterial activity against *H. pylori*, *M. tuberculosis* and *M. avium* at concentrations lower (MIC = 3.25 μg/ml; MIC = 50 μg/ml; MIC = 62.5 μg/ml, respectively) than those which showed cytotoxicity in normal human fibroblasts cells (IC_{50} = 81.32 μg/ml). Possibly, synergistic interactions among volatile oils components which are beneficial to their different biological properties (Raut and Karuppayil, 2014).

Conclusions

It is clear that studies of volatile oils from propolis are far from being exhaustive. Further research is needed to reveal their chemistry and to support their medicinal properties scientifically. Differences in the chemical composition of volatile oils extracted from propolis are observed as the result of their botanic and geographic origin. Acetophenone, nerolidol, β-caryophyllene, spathulenol and δ-cadinene were the major constituents identified in EOP. Evaluation of the antibacterial activity of EOP showed that it is significant against the bacteria *H. pylori*, *M. tuberculosis* and *M. avium*. The preliminary result of the antioxidant potential showed by EOP could be promising for the development of news cosmetics with antioxidant potential. In assays of cytotoxic activity, EOP proved to be cytotoxic against all cell lines under study, but it showed no selectivity to tumor cell lines. The hydrodistillation process under study was capable of extracting bioactive substances from brown propolis and originate the volatile oil which is responsible for biological properties found by this study. In short, further *in vivo* experiments are needed to confirm the absence of potential mutagenic risks posed by EOP from the Brazilian Atlantic Forest biome and its mechanism of action.

Authors' contributions

VHML, KCRA, CCFA, MLR, JMS, ABR and ISS contributed to the design and implementation of the experiment. AEMC, DCT and CHGM proofread the manuscript. MLDM contributed to the statistical analyses and also proofread the manuscript. MLDM and AEMC contributed to the chromatographic analysis. CHGM and DCT, as professors, worked on overall planning and experiment design. All authors contributed to biological studies, laboratory experiments and data analyses. They proofread the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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