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Artigo

Analysis of a Brazilian green propolis from *Baccharis dracunculifolia* by HPLC-APCI-MS and GC-MS

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RESUMO: "Análise de uma própolis verde brasileira da *Baccharis dracunculifolia* por CLAE-ICPA-EM e CG-EM". Os extratos em etanol e diclorometano de uma própolis verde de *Baccharis dracunculifolia* foram analisados por CLAE-ICPA-EM e CG-EM, respectivamente. A técnica de CLAE-EM-ICPA, no modo positivo, forneceu uma completa e inequívoca composição química da amostra de própolis verde. Ela serve como impressão digital para amostras diferentes de própolis. A composição do extrato em etanol consistiu fundamentalmente de ácido cinâmico e derivados, flavonóides, ácido benzóico e alguns benzoatos, aromáticos não hidroxilados, e ácidos e ésteres alifáticos, os quais são normalmente ignorados na literatura porque não absorvem luz UV. Os constituintes principais do extrato em diclorometano foram compostos prenilados, alcanos e terpenóides.

Unitermos: *Baccharis dracunculifolia,* Asteraceae, própolis, própolis verde, CLAE-EM-IQPA, CG-EM.

ABSTRACT: Ethanol and dichloromethane extracts of a Brazilian green propolis from *Baccharis dracunculifolia* were analyzed by HPLC-APCI-MS and GC-MS, respectively. The HPLC-APCI-MS technique, at the positive mode, furnished a complete and unequivocal chemical composition of the green propolis sample. It serves as fingerprint for different propolis samples**.** The composition of the ethanol extract consisted mainly of cinnamic acid and derivatives, flavonoids, benzoic acid and a few benzoates, non-hydroxylated aromatics, and aliphatic acids and esters, which are normally not reported in the literature because they do not absorb UV light. The main constituents of the dichloromethane extract were prenylated compounds, alkanes and terpenoids.

Keywords: *Baccharis dracunculifolia*, Asteraceae, propolis, green propolis, HPLC-APCI-MS**,** GC-MS.

INTRODUCTION

Propolis is a resinous material produced by honeybees from vegetable resins, exudates, wax, pollen, leaf pieces, and self-secretion. It is used to seal and to protect the hive against heat, cold, wind, water, insects and microorganisms. (Ghisalberti, 1979; Rohwedder and Hausteen, 1987; Neto et al., 2002).

Propolis ethanol extracts have been used in the popular medicine for different purposes (Niraldo, et al., 2006; Soares et al., 2006; Tavares et al., 2006; Lemos et al., 2007; Missima and Sforcin, 2008; Simões et al., 2008). Flavonoids contained in European propolis were considered the constituents that had the beneficial action on the human organism. However in the last recent years many propolis other than the European ones have demonstrated therapeutic properties and they do not contain or contain insignificant amount of flavonoids (Park et al., 2000; Pereira et al., 2002; Longhini et al., 2007; Sousa et al., 2007).

Review publications report that over a hundred compounds were already identified in propolis (Greenaway et al., 1991; Marcucci, 1996; Lustosa et al., 2008). The propolis analysis is a very difficult task because their composition changes according to the region, season and existing flora in addition to the inherent difficulties associated to analysis of complex mixtures from vegetal sources. In spite of that, various researchers demonstrated that *Baccharis dracunculifolia* is the main vegetable source of Brazilian green propolis (Bankova et al., 1999; Bastos et al, 2000; Kumazawa et al., 2003; Park et al., 2003, 2004, 2005). Further, recently, a chemical marker for propolis from *Baccharis dracunculifolia* was proposed (Nascimento et al.,

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2008).

By means of derivatization with BSTFA, GC-MS analysis and a lot of reference compounds, over a hundred compounds could be identified in a propolis sample (Greenaway et al., 1991). The limitations of this technique reside on a more complex mixture after derivatization, the need of many rare reference compounds and the presence of non-volatile or underivatizable compounds that cannot be detected accordingly. Even using high temperatures (near 400 ^{o}C), this technique has limitations (Neto et al., 2002)

Actually the tedious work on column fractionations and isolation of unknown compounds is giving place to high performance liquid chromatography (HPLC), which can provide a quickly separation of the components, quantification and information related to its UV spectrum. When coupled to Infrared (IR), Nuclear Magnetic Resonance (NMR) or Mass Spectrometry (MS), the HPLC can expand much more its analysis range. Especially the HPLC-MS technique is very useful because it avoids the limitations imposed by the UV detection while detecting also the molecules that do not absorb in the UV region. Today the HPLC-MS can use modern revolutionary techniques like ESI (electrospray ionization), APCI (atmospheric pressure chemical ionization), MALDI (matrix-assisted laser desorption/ionization), etc. that volatilize organic molecules of low and high molecular weights having medium to high polarity (Hoffmann and Stroobant, 2001).

Some of these new techniques were recently employed on propolis analysis (Valcic et al., 1999; Midorikawa et al., 2001; Pietta et al., 2002; Kumazawa et al., 2003).

Due to the broad use in the medicine, actually the propolis investigation work is focused on ethanol extracts. Many model compounds were used to investigate samples of Brazilian propolis by HPLC-ESI-MS at the negative mode (Midorikawa et al., 2001). Based on the retention times and mass spectra of forty-one standards, these authors identified a lot of components in the propolis samples. Sawaya and collaborators also used the HPLC-ESI-MS at negative mode to identify eight markers in Brazilian propolis from different origins (Sawaya et al., 2004). Finally, using the same technique and others procedures, Kumazawa and collaborators compared propolis ethanol extracts from fourteen different countries (Kumazawa et al., 2004).

In the present work an attempt to expand the range of identification of the constituents of a propolis ethanol extract is presented. HPLC-APCI-MS at the positive mode was used and the identification was based on mass spectrometry, standards, UV spectrum, retention times, Kovat's indexes (Adams, 2001), the publications cited above and others (Nascimento and Bezzan, 2001; Neto et al., 2002; Nascimento et al., 2003a; Nascimento et al., 2003b; Nascimento et al., 2008; Negri et al., 2003).

Finally, a GC-MS investigation of the dichloromethane extract of the same propolis was also carried out in order to complement the data acquired from HPLC-APCI-MS.

MATERIAL AND METHODS

Propolis sample

The Santa Barbara Apiary located in the State of Minas Gerais supplied the sample of green propolis.

Table 1. Components of the EEP related to Figure 1.

Retention time (min)	Compound
18.3	ethyl cinnamate
20.2	benzyl caffeate
22.1	cinnamyl caffeate
24.0	cinnamyl coumarate
25.8	dihydrocinnamyl ferulate
27.1	pinobanksin
29.6	kaempferol
32.8	2,2-dimethyl-6-cromene-6-propenoic acid, 3-prenyl-p-coumaric acid
34.0	methyl 2,2-dimethyl-6-chromene-propenoate
34.6	diprenyl coumarate and n.i. (λ 238, 318 nm)
35.5	pinobanksin-3-acetate, kaempferide, dihydrokaempferide
36.0	ermanin
36.6	capillartemisin A
37.3	(E)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid
38.2	(E)-3-[2,3-dihydro-2-(1-hydroxy-1-methylethyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid
38.9	artepillin C
41.0	n.i. (λ 220, 286 nm) and n.i. (λ 235, 278 nm)
41.9	n.i. (λ 262, 318 nm)

n.i.: not identified.

It was collected in October and is representative of green propolis from *Baccharis dracunculifolia* as comproved by several analyses of green propolis samples from different regions. The ethanol extract of propolis (EEP) was obtained by mixing 3.00 g of sample with 10.0 mL of ethanol (Vetec brand, analytical grade, 95 %). The mixture was stirred for 24 hours away from light and then filtered and put into a freezer to separate the wax. A new filtration gave the studied extract. The dichloromethane extract of propolis (DEP) was obtained using another 3.00 g sample as above, except on that it was not put into the freezer for wax separation.

Gas chromatography coupled to mass spectrometry (GC-MS)

The analysis was performed in a gaseous chromatograph by Shimadzu, model GC-17A, equipped with a DB-5 30 meters capillary column, 0.25 mm of i.d., 0.25 μm of film thickness, coupled to a mass spectrometer of same brand, model GCMS-QP5000, equipped with database of 330.000 mass spectra. The runs were performed under the following conditions: initial column temperature: 60 °C, injector at 220 °C and interface at 240 °C; column program: 60-240 °C at 3 °C/min, 20 min at 240 °C. 1 μL of the sample of was injected under helium as carrier gas. The mass spectrometer worked under impact energy of 70 eV and the mass detection included molecules from 40 to 450 Da.

High performance liquid chromatography coupled to mass spectrometry (HPLC-MS)

The HPLC runs were performed on a Shimadzu Class VP series liquid chromatograph equipped with diode array detector (PDA) and a reversed-phase CLC-ODS 30 cm column. The gradient conditions used were methanol HPLC grade and water/formic acid (0.5%), starting with methanol 20% and after 40 minutes, 100% methanol, in a 60 minutes run. The injected volume was 20 μL.

The high performance liquid chromatography coupled to atmospheric pressure chemical ionization and mass spectrometry (HPLC-APCI-MS) analysis, at the positive mode, was carried out in a Quattro LC-Micromass Mass Spectrometer. The chromatogram presented in this work (Figure 2) is the Total Ions Chromatogram, TIC.

Identification of the propolis constituents

The identification of the propolis components was based on a long and tedious work with standards, mass spectra library (Wiley 7), private UV spectra library, retention times, Kovat's indexes, and literature. As the compounds were not isolated and directly compared with standards, it is not warranted that they really are the assigned ones.

RESULTS AND DISCUSSION

Figure 1 presents the HPLC chromatogram of EEP. Low retention time components (less than 15 minutes), as chlorogenic, gallic, benzoic and caffeic acids, and vanillin are practically absent. This is not in agreement with HPLC results reported by Park and collaborators (Park et al., 2004) as well as Midorikawa and collaborators (Midorikawa et al., 2001). Both groups worked with ethanol extracts of green propolis

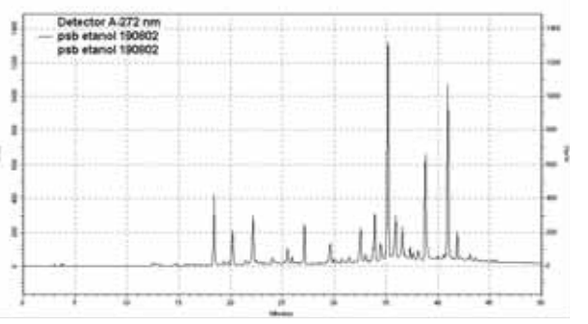

Figure 1. HPLC chromatogram of the ethanol extract of propolis.

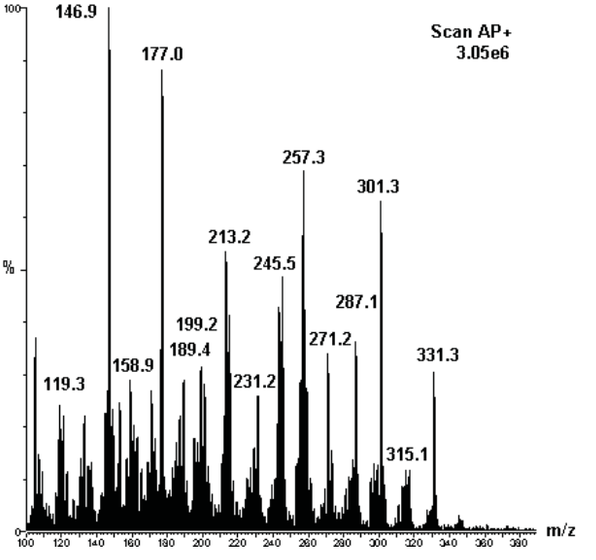

Figure 2. APCI-MS chromatogram of the ethanol extract of propolis.

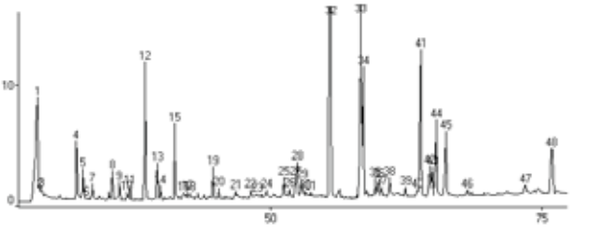

Figure 3. GC chromatogram of the dichloromethane extract of propolis.

Table 2. Compounds of the EEP related to Figure 2.

Nº	M	Compound	% base-peak
1	330	pinobanksin-3-acetate	30
2	316	(E)-3-[2,3-dihydro-2-(1-hydroxy-1-methylethyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid	12
3	314	ermanin	12
4	312	dihydrocinnamyl ferulate	9
5	302	dihydrokaempferide	9
6	300	artepillin C, kaempferide, 4-hydroxi-3,5-diprenylcinnamic acid	63
7	298	(E)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid	12
8	296	cinnamyl caffeate	13
9	294	methyl linoleate	10
10	286	kaempferol	35
11	284	acacetin, stearic acid	14
12	282	oleic acid	10
13	280	linoleic acid	7
14	272	pinobanksin (chalcone)	16
15	270	benzyl caffeate	33
16	268	cinnamyl coumarate	7
17	260	14-acetoxy-trementone ?	10
18	258	2',4',6'-triidroxi-chalcone	27
19	256	palmitic acid, allyl 3-prenylcinnamate	68
20	254	benzyl coumarate	29
21	252	methyl-flavonol	13
22	244	methyl 2,2-dimethyl-6-chromene-propenoate	48
23	242	methyl miristate	42
24	240	hydroxy-flavanone	10
25	238	flavonol	9
26	232	3-prenyl-p-coumaric acid	7
27	230	2,2-dimethyl-6-cromene-6-propenoic acid	26
28	228	miristic acid	17
29	226	phenylethyl benzoate	13
30	224	flavanone	10
31	218	methyl 2,2-dimethyl-6-chromene-carboxylate	6
32	216	n.i.	10
33	214	methyl laurate	42
34	212	n.i.	53
35	210	dihydrochalcone	15
36	204	2,2-dimethylchromene-6-carboxilic acid, sesquiterpenes	7
37	202	trementone	13
38	200	lauric acid	28
39	198	ethyl gallate	31
40	196	methyl veratrate	16
41	194	ferulic acid	17
42	190	n.i.	9
43	188	5-(2,5-dimethylphenyl)-2(3H)-furanone	28
44	186	methyl decanoate	22
45	184	methyl gallate	17
46	182	veratric acid	12
47	180	caffeic acid	8
48	178	4-hydroxy-3-methoxycinnamaldehyde	7
49	177	n.i.	10
50	176	ethyl cinnamate	87
51	174	5-phenyl-pentadien-2,4-oic acid	8
52	172	decanoic acid	17
53	170	gallic acid	27
54	168	vanillic acid	13
55	166	dihydrocoumaric acid	6
56	164	*p*-coumaric acid	11

Nº	M	Compound	% base-peak
57	162	methyl cinnamate	17
58	160	pimelic acid, ethyl glutarate	20
59	158	isopentyl isobutirate, isopentyl butanoate, pelargonic acid	28
60	156	n.i.	13
61	152	4-methoxy-benzoic acid	24
62	150	dihydrocinnamic acid	8
63	148	cinnamic acid	23
64	146	ethyl succinate	100
65	144	octanoic acid, isobutyl isobutirate, isobutyl butirate	22
66	142	n.i.	7
67	136	methyl benzoate	13
68	134	malic acid	12
69	132	ornitine	23
70	130	isopentyl acetate, 2-methylbutyl acetate	13
71	126	6-methyl-hept-5-3-en-2-one	6
72	122	benzoic acid	12
73	120	acetophenone, 2,3-benzo-furan	23
74	118	succinic acid	25
75	116	isobutyl acetate	7
76	108	benzyl alcohol	12
77	106	benzaldehyde	15
78	104	styrene	37

n.i.: not identified.

but the ethanol concentration was 80 %, which allows better dissolution of more hydrophilic compounds.

The peaks observed at the chromatogram belong practically to cinnamic acid derivatives and flavonoids (Table 1) and are in good agreement with the results obtained elsewhere (Pietta et al., 2002; Midorikawa et al., 2001). The prenylated compounds are very important constituents of green propolis (Vasconcelos, 2006; Pereira et al., 2202, Bankova et al., 1999).

Figure 1 is a good example for the HPLC technique constraints. Even using diode array detector the number of detected components is relatively low. This is due to different absorbance of the constituents (the apparatus is automatically calibrated according to the higher absorbance), no detection of compounds that do not absorb UV light, and peak overlapping.

Figure 2 presents the APCI-MS chromatogram of the EEP. Using the positive mode, the molecular ions appear as [M + 1]. Comparing Figure 2 with Figure 1, it is evident that this new technique gives much more information about the extract than the HPLC does. The number of detected compounds is incomparably greater and includes much more molecules, which absorb or not UV light. This chromatogram is the best indirect view of the extract composition and can be used as fingerprint of it. Certainly an APCI-MS chromatogram of another propolis ethanol extract will be different.

In Figure 2, the detected mass range goes from circa 100 Da to 350 Da, confirming the absence of chlorogenic acids and other high molecular weight compounds as dimeric coniferyl acetate (M=442), propolis benzofurans A (M=454) and B (M=438), (E)-3-(2,3-dihydro-2-(1-methyethenyl)-7-prenyl-5-benzofuranyl-2-propenoic acid (M=448), etc. and also the insignificant presence of small molecules (Midorikawa et al., 2001).

Table 2 shows the identified and not identified compounds corresponding to Figure 2, with the respective percentage (TIC). Only the compounds whose concentration was greater than 5 % of the base peak were took in account.

For the first time a complete table showing the compounds present in the propolis ethanol extract is shown. The main constituents were cinnamic acid and derivatives, flavonoids, benzoic acid and a few benzoates, non-hydroxylated aromatics, and aliphatic acids and esters. The aliphatic compounds are normally not reported in the literature because they do not absorb UV light. It is very important to note that the presence of compounds that do not absorb UV light is significant. All of the compounds of Table 2 have already been reported in the propolis literature but a lot of them were not reported in ethanol extracts. Allyl 3-prenylcinnamate, which was isolated from chloroform extract of *Baccharis dracunculifolia* green propolis (Negri et al., 2003), has been recently proposed as chemical marker for this kind of propolis (Nascimento et al., 2008).

Figure 3 presents the GC-MS chromatogram of the DEP and Table 3 shows the identified and not identified volatile compounds of this extract. The main constituents are prenylated compounds (over 50 %) followed by terpenoids, which gives the special odor

Table 3. Components of the DEP having concentration ≥ 0.27% (related to Figure 3).

Peak number	Retention time (min)	Molar mass	Compound	% TIC
1 + 2	28.76	150	dihydrocinnamic acid	7.55
3	29.05	178	dihydrocinnamic acid, ethyl ester	0.56
4	32.22	222	dihydrocinnamic acid, TMS ester (contamination by BSTFA)	2.14
5	32.82	204	*trans*-caryophillene	0.94
6	33.18	204	*trans*-*alfa*-bergamotene	0.39
7	33.69	204	aromadendrene	0.54
8	35.51	204	germacrene-D	0.75
9	36.19	204	bicyclogermacrene	0.58
10	36.87	204	*alfa*-muurolene	0.30
11	37.15	204	*gamma*-cadinene	0.39
12	38.54	204	*trans*-nerolidol	3.64
13	39.68	220	(+)-spathulenol	0.93
14	39.97	222	globulol	0.40
15	41.23	188	5-(2,5-dimethylphenyl)-2(3H)-furanone	2.26
16	42.07	220	(-)-spathulenol	0.41
17	42.38	202	desmethoxy encecalin	0.33
18	42.68	222	*alfa*-cadinol	0.34
19	44.75	222	2-*cis*,6-*trans*-farnesol	0.87
20	45.29	220	14-hydroxy-*alpha*-humulene	0.39
21	46.86	164	*p*-coumaric acid	0.59
22	48.21	236	n.i.	0.32
23	48.86	218	xanthorrihzol	0.29
24	49.70	220	sesquiterpene alcohol	0.40
25	51,27		n.i.	0.75
26	51.83		n.i.	0.34
27	52.31	220	sesquiterpene alcohol	0.72
28	52.52	230	n.i.	1.19
29	52.95	308	*p*-coumaric acid, di-tms ester (contamination by BSTFA)	0.65
30	53.30	256	palmitic acid	0.28
31	53.74		n.i.	0.36
32	55.53	256	allyl 3-prenylcinnamate	40.28
33	58.34	270	prenylated compound	5.35
34	58.59	270	prenylated compound	3.47
35	59.72		n.i.	0.58
36	59.97	270	prenylated compound	0.74
37	60.31	254	benzyl coumarate	0.34
38	61.03	272	n.i.	1.13
39	62.47	268	n.i.	0.35
40	63.56		n.i.	0.31
41	63.88	270	prenylated compound	6.32
42	64.74		n.i.	1.16
43	64.98	310	docosane	1.06
44	65.32		n.i.	2.79
45	64.24		n.i.	3.08
46	68.19		n.i.	0.27
47	73.50	338	tetracosane	0.64
48	75.90	352	pentacosane	2.50

n.i.: not identified.

to the green propolis. Allyl 3-prenylcinnamate is longer the more abundant and until today was detected only in the *Baccharis dracunculifolia* resin and therefore was proposed as chemical marker for this kind of propolis (Nascimento et al., 2008).

Taking in account that the ethanol and dichloromethane extracts solubilized 36.61 % and 34.21 % of the propolis, respectively, a comparison of Tables 1, 2 and 3 brings out interesting results. The volatile terpenoids and alkanes are present in the ethanol extract as minorities and were not considered in Tables 2 and 3. This extract fundamentally contains polar molecules

that are not volatile. However, the analysis of volatile molecules by GC is very important to classify the aroma and plant sources of the propolis. Actually, the analysis of both extracts, EEP and DEP, are complementary for a better propolis chemical investigation.

CONCLUSION

The HPLC-APCI-MS technique, at the positive mode, provided a complete and unequivocal chemical composition of a green propolis sample. It serves as fingerprint for different propolis samples. The main constituents of the 95 % ethanol extract of the green propolis from *Baccharis dracunculifolia* were cinnamic acid and derivatives, flavonoids, benzoic acid and a few benzoates, non-hydroxylated aromatics, and aliphatic acids and esters. The main constituents of the dichloromethane extract were prenylated compounds, alkanes and terpenoids. By means of this technique it was possible to detect the showed aliphatic compounds which are normally not reported in the literature because they do not absorb UV light.

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