Buds of *Baccharis dracunculifolia*: potent source of biologically active caffeoylquinic acids and labdane-type diterpenes of Brazilian propolis

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Abstract

Liquid chromatography mass-spectrometry (LC-MS) analysis of Brazilian propolis and the leaves, buds, stem and flowers of *Baccharis dracunculifolia* were performed. The water extracts of propolis and *B. dracunculifolia* both contain 3,4-di-*O*-caffeoylquinic acid (**28**), 3,5-di-*O*-caffeoylquinic acid (**31**) and chlorogenic acid (**32**). The intensities of **28** and **31** in the water extract of the buds were stronger than the other parts, suggesting that the buds of *B. dracunculifolia* should be potent source of these caffeoylquinic acids. Moreover, 27 individual compounds including labdane-type diterpenes, prenylated compounds, flavonoids and other phenolics which were previously isolated from Brazilian propolis, were also identified in the MeOH extract of *B. dracunculifolia*. Among them, 24 compounds were detected in the buds alone, indicating that it should be an important source of Brazilian propolis. The botanical origin of 19 components of Brazilian propolis was, for the first time, established to be *B. dracunculifolia*.

Key words Propolis, *Baccharis dracunculifolia*, Caffeoylquinic acids, Labdane-type diterpenes, Cinnamic acid derivatives, Prenylated compounds.

Introduction

Propolis, a resinous hive product collected by honeybees from various plant sources, contains sticky plant substances mixed with bee wax and other bee secretions. It has a pleasant aromatic odor and is yellow-green to dark brown in color. Propolis is widely used in traditional medicine and is reported to have a broad spectrum of pharmacological properties.[1,2] Besides its uses in traditional medicine, it has gained popularity as a health drink and is used extensively in food and beverages in various parts of the world including Japan, the United States and Europe, where it is claimed to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer.

The composition of propolis depends upon the vegetation of the area from where it was collected. Propolis from temperate zones contains predominantly phenolic compounds including flavonoids and cinnamic acid derivatives.[3] Diterpenes, prenylated compounds and dicaffeoylquinic acids, which are virtually absent in temperate propolis, were reported from the tropical propolis of the South-American continent together with lignans, flavonoids and other classes of compounds.[4] The difference in the composition of propolis from temperate and tropical zones is mainly due to the difference of the vegetation. The poplar tree (*Populus nigra*) is considered as dominant plant source of the temperate zone (Europe) because of their wide distribution and presence of various phenolic components found in propolis.[5] In the recent days, several groups of researchers focused their attention on Brazilian propolis, because of its growing market and interesting biological properties. As a consequence, more than 100 components have been identified so far from Brazilian propolis and among them coumaric acid, ferulic acid, dihydrocinnamic acid, pinobanksin, kaempferol, apigenin, pinocembrin, kaempferide, 3,5,7-

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trihydroxy-4'-methoxyflavanol ether and fatty substances including α-pinene, β-pinenefarnesol, butanedioic acid and hexadecanoic acid were reported to derive from four different plants, *i.e., Araucaria angustifolia, Baccharis dracunculifolia, Hyptis divaricata* and *Eucalyptus* sepecies.[4,6-10] But these reports could not explain the origin of several other components present in Brazilian propolis. In this paper, we report the LC-MS analysis of Brazilian propolis and different parts of *Baccharis dracunculifolia* to elaborate the origin of caffeoylquinic acid derivatives, labdane-type diterpenes and phenolics of Brazilian propolis.

Materials and Methods

Chemicals: 3-Hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid (**1**), 2,2-dimethyl-8-prenylchromene-6-propenoic acid (**2**), 2,2-dimethylchromene-6-propenoic acid (**3**), 2,2-dimethylchromene-6-carboxylic acid (**4**), artepillin C (**5**), 4-dihydrocinnamoyloxy-3-prenylcinnamic acid (**6**), 4-hydroxy-3-prenylcinnamic acid (**7**), vanillin (**8**), coniferyl aldehyde (**9**), isocupressic acid (**10**), 15-acetoxyisocupressic acid (**11**), agathic acid (**12**), agathic acid 15-methyl ester (**13**), agathalic acid (**14**), cupressic acid (**15**), tremetone (**16**), viscidone (**17**), 12-acetoxyviscidone (**18**), betuletol (**19**), kaempferide (**20**), ermanin (**21**), dihydrokaempferide (**22**), dimeric coniferyl acetate (**23**), (*E*)-3-[4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl]-5-prenylphenyl]-2-propenoic acid (**24**), (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid (**25**), propolis-benzofuran A (**26**) and propolis-benzofuran B (**27**) were isolated from the MeOH extract,[10,11] while 3,4-di-*O*-caffeoylquinic acid (**28**), methyl 3,4-di-*O*-caffeoylquinate (**29**), methyl

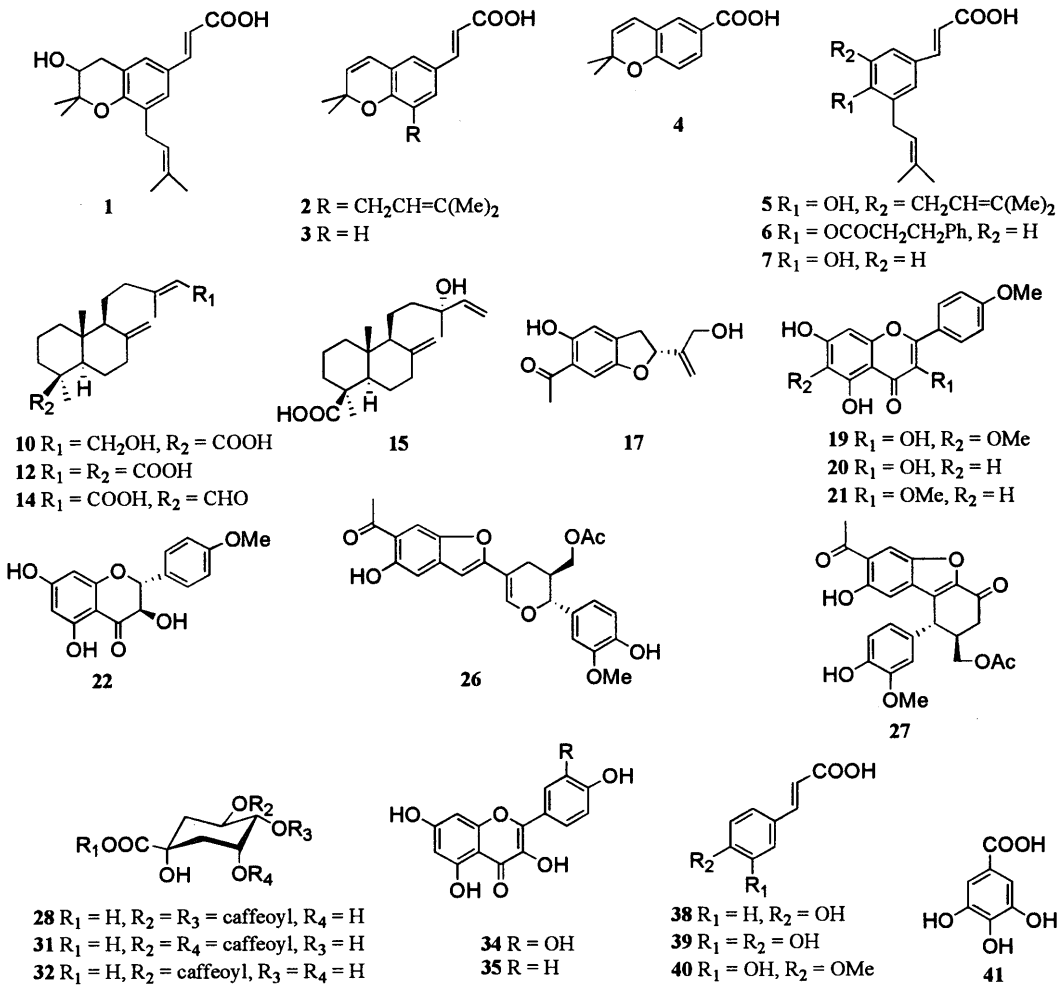

Fig. 1 Structure of the compounds detected in *B. dracunculifolia*.

4,5-di-*O*-caffeoylquinate (**30**) and 3,5-di-*O*-caffeoylquinic acid (**31**) were obtained from the water extract of Brazilian propolis.[12] Chrysin (**33**), kaempferol (**35**), quercitrin (**36**), caffeic acid (**39**) and ferulic acid (**40**) were purchased from Wako (Tokyo, Japan); quercetin (**34**), cinnamic acid (**37**) and *p*-coumaric acid (**38**) were from Nacalai Tesque (Kyoto, Japan); and chlorogenic acid (**32**) was from Tokyo Kasei (Tokyo, Japan). Gallic acid (**41**) was isolated from *Rhodiola sacra*.[13]

Propolis and plant material: The Green propolis (No. TMPW 19917) and the different parts of *B. dracunculifolia* (Herbarium No. SK-1) were collected from Minas Gerais, Brazil in 1999 and in 2001, respectively. The voucher specimens are preserved in the Museum for Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan, as a reference.

Preparation of extract: A small piece of propolis (10 g) was extracted with 100 ml of H_2O at 80 ℃ for 3 hrs to give a H_2O extract (1.42 g). The residue was further extracted with MeOH (100 ml) under reflux for 3 hrs giving a MeOH extract (0.28 g). The dried flowers (176 mg) of *B. dracunculifolia* were extracted with MeOH (20 ml×2) under reflux, and then with H_2O (20 ml×2) at 80 ℃, giving MeOH (46.8 mg) and H_2O (14.2 mg) extracts, respectively. The buds (870 mg), leaves (893 mg) and stem (1014 mg) of *B. dracunculifolia* were also extracted with the same procedure using an equal volume of solvent to yield MeOH extracts (359 mg, 320 mg and 196 mg, respectively) and water extracts (46 mg, 57 mg and 21 mg, respectively).

LC-MS analysis: The LC-MS analyses were performed using a JEOL (Tokyo, Japan) JMS-700T four-sector mass spectrometer, coupled to a Hewlett Packard (Waldbronn, Germany) model HP1100 HPLC system.[14] The MS was operated in the negative atmospheric pressure chemical ionisation (APCI) mode [capillary temperature - 400℃; ion injection time - 0.1 s; column - Shim-pack (Shimadzu, Kyoto, Japan) CLC-ODS (150 × 6.0 mm i.d.); column temperature - 40℃; mobile phase - linear gradient of 0.5% acetic acid: methanol from 70:30 (v/v) to 20:80 in 30 min; flow rate - 1 ml/min]. Each sample was dissolved in HPLC grade water or methanol (5 mg/mL), filtered using a Millipore (Millipore, Bedford, MA, USA) SJLG 250 filters, and aliquots of the filtrate (10 μl) were injected for analysis.

Results and Discussion

The leaves, stem, buds and flowers of *B. dracunculifolia* collocated from Minas Gerais, Brazil, were successively extracted with MeOH and water. All these extracts were subjected to LC-MS analysis. The negative APCI mode was applied, in which 41 standard samples including prenylated compounds (**1**, **2**, **5-7**, **24**, **25**), labdane-type diterpenes (**10-15**), caffeoylquinic acids (**28-32**) and phenolic compounds gave $[M-H]^-$ ions. The standard samples were well separated on the applied HPLC condition with few exceptions (Table I and II). Compounds having identical retention time were identified on the basis of their mass spectrum.

The water extract of propolis showed four major

Table I LC-MS data of the water extract of Brazilian propolis and different parts of *B. dracunculifolia*

RT (min)[a]	Compd	*m/z*	propolis	*B. dracunculifolia*			
				Flower	Bud	Leaf	Stem
3.60	**41**	169	-	-	-	-	-
5.25	**32**	353	++	++	++	++	+
7.00	**39**	179	+	-	-	-	-
8.45	**8**	151	-	-	-	-	-
9.60	**38**	163	+	-	-	-	-
10.15	**31**	515	++	++	+++	+++	++
10.15	**40**	193	-	-	-	-	-
11.05	**9**	177	-	-	-	-	-
11.90	**28**	515	+++	++	**	+++	++

-, not detected; +, relative intensity less than 10% in TIC; ++, relative intensity less between 50% in TIC; +++, relative intensity more than 50% in TIC; ** relative intensity is 100% in TIC;[a] retention time (RT) of the standard sample.

Table II LC-MS data of the water extract of Brazilian propolis and different parts of *B. dracunculifolia*

RT (min)[a]	Compd	*m/z*	propolis	*B. dracunculifolia*			
				Flower	Bud	Leaf	Stem
3.60	**41**	169	-	-	-	-	+
5.25	**32**	353	-	+	-	+	-
7.00	**39**	179	+	-	+	+	+
8.45	**8**	151	-	-	-	-	-
9.60	**38**	163	+	-	+	+	-
10.15	**31**	515	+	++	+	++	+
10.15	**40**	193	-	++	+	++	+
11.05	**9**	177	-	-	-	-	-
11.90	**28**	515	+	++	++	++	++
14.20	**36**	463	-	-	-	-	-
15.95	**23**	441	-	-	-	-	-
15.95	**29**	529	-	-	-	-	-
17.40	**17**	233	-	-	+	++	+
17.85	**34**	301	-	++	-	-	-
18.40	**37**	147	-	-	-	-	-
18.90	**22**	301	++	++	+++	++	+++
21.25	**35**	285	+	-	++	++	++
22.55	**27**	437	-	-	++	-	++
22.55	**30**	529	-	-	-	-	-
24.20	**18**	275	-	-	-	-	-
25.69	**7**	231	++	++	+++	++	+++
26.39	**4**	203	++	-	++	-	++
27.34	**33**	253	+	-	-	-	-
27.89	**3**	229	-	-	++	-	++
27.89	**16**	201	-	-	-	-	-
28.54	**20**	299	++	++	+++	++	+++
28.84	**19**	329	++	+	+++	++	+++
29.39	**21**	313	++	-	+++	++	+++
30.99	**25**	297	-	-	-	-	-
31.99	**26**	451	-	-	+	-	-
32.34	**1**	315	+	+	+	+	-
33.94	**12**	333	++	-	+++	-	-
33.94	**5**	299	++	+	++	+	++
33.94	**10**	319	++	-	+	-	-
33.94	**14**	317	++	-	+	-	++
34.34	**15**	319	-	-	++	-	-
37.94	**24**	447	+	-	-	-	-
38.54	**6**	363	+	-	+	-	+
40.54	**2**	297	+	-	++	-	++
41.94	**11**	361	-	-	-	-	-
41.94	**13**	347	-	-	-	-	-

-, not detected; +, relative intensity less than 10% in TIC; ++, relative intensity less between 50% in TIC; +++, relative intensity more than 50% in TIC.[a] retention time (RT) of the standard sample.

peaks corresponding to chlorogenic acid (**32**), 3,4-di-*O*-caffeoylquinic acid (**28**), 3,5-di-*O*-caffeoylquinic acid (**31**) and *p*-coumaric acid (**38**) in its total ion chromatogram (TIC) and caffeic acid (**39**) as a minor peak (Fig. 2). The water extracts of flowers, buds, leaves and stem of *B. dracunculifolia* also showed peaks corresponding to **28**, **31** and **32**, suggesting that they should be derived from *B. dracunculifolia*. The intensities of the peaks corresponding to **28** and **31** (Table I) in the water extract of buds were stronger than the leaves, stem and flowers indicating that there high content in the buds. To the best of our knowledge, it was the first report of chlorogenic acid (**32**) in *B. dracunculifolia*.

The TICs of the MeOH extract of both propolis and *B. dracunculifolia* were more complicated (Fig. 2) than their water extracts. Compounds **28**, **31**, **38** and **39** found in the water extract were also detected in MeOH extract of propolis. Moreover, prenylated compounds (**1**, **2**, **5**, **6**, **7** and **24**), flavonoids (**19**-**21** and **33**) and labdane-type diterpenes (**10**, **12** and **14**) were also detected in MeOH extract of propolis, which are considered to be typical compounds of Brazilian propolis (Table II). Similarly, 3,4-di-*O*-caffeoylquinic acid (**28**) and 3,5-di-*O*-caffeoylquinic acid (**31**), ferulic acid (**40**), flavonoids (**19**, **20** and

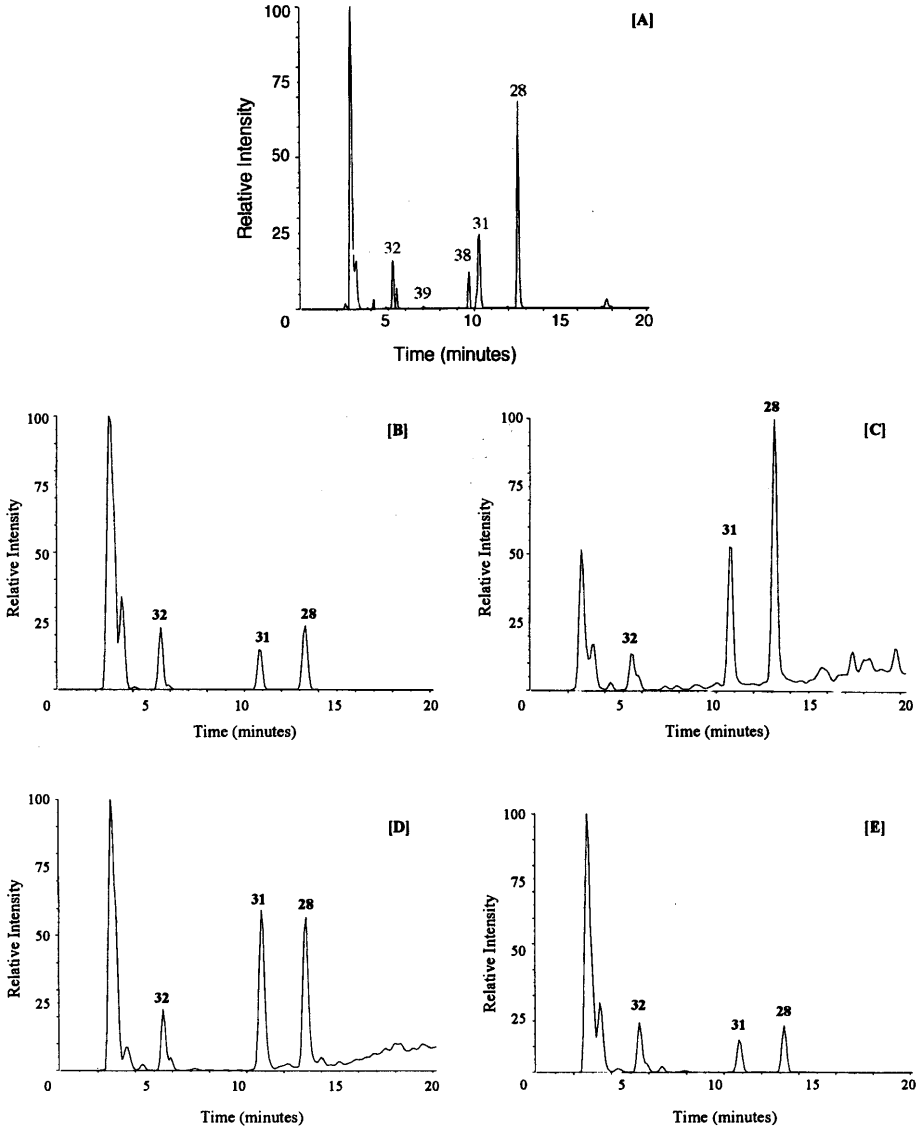

Fig. 2 TIC of the water extracts of propolis [A] and the different parts of *B. dracunculifolia*. [B] Flower, [C] Buds, [D] Leaves and [E] Stem

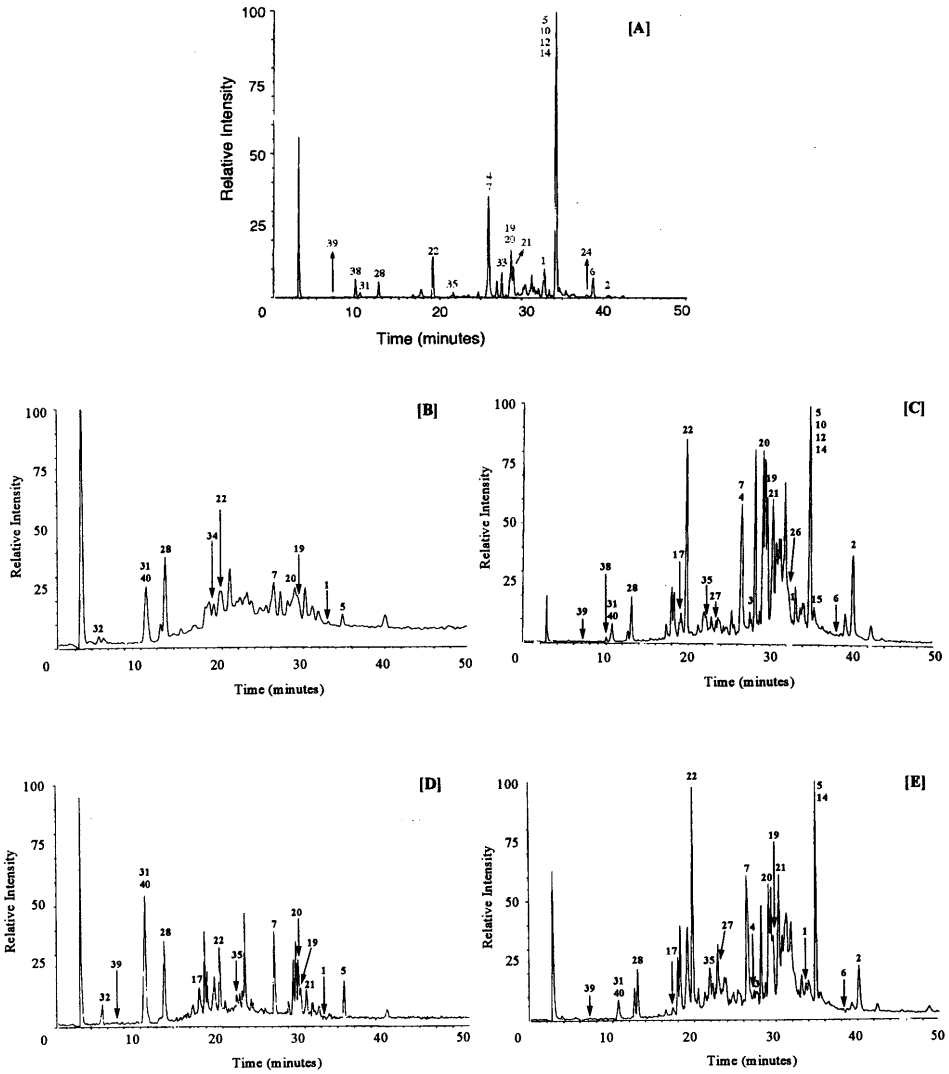

Fig. 3 TIC of the MeOH extracts of propolis [A] and the different parts of *B. dracunculifolia*. [B] Flower, [C] Buds, [D] Leaves and [E] Stem

22), artepillin C (**5**) and 4-hydroxy-3-prenylcinnamic acid (**7**) were detected in all the MeOH extracts of *B. dracunculifolia*. Interestingly, the diterpenes isocupressic acid (**10**), agathic acid (**12**), agathalic acid (**14**) and cupressic acid (**15**) were also detected in the MeOH extract of the buds together with 20 other compounds. All these diterpenes were detected for the first time *in B. dracunculifolia*. It is worthwhile to mention here that 27 individual components previously isolated from Brazilian propolis were detected in different parts of *B. dracunculifolia* and among them 24 were found to be present in the buds.

Moreover, the TIC pattern of the MeOH extract of propolis was found to be similar to those of the MeOH extract of the buds, except for the difference in intensities of individual peaks (Fig. 3). These results suggested that the buds of *B. dracunculifolia* should be a potent source of Brazilian propolis. The isolation of four dicaffeoylquinic acid derivatives (**28**, **29**, **31** and 4,5-di-*O*-caffeoylquinic acid) and several other phenolic compounds including flavonoids, cinnamic acid derivatives, diterpenes and their glucosides from the aerial parts of *B. dracunculifolia* by Nagatani *et al.* further supported our finding that *B. dracunculifolia* is a potent source of caffeoylquinic acids and labdane-type diterpenes of Brazilian propolis.[15-17]

Considering the pharmacological activities of Brazilian propolis, labdane-type diterpenes, caffeoylquinic acid derivatives and prenylated compounds together with flavonoids come as active constituents.[4] The caffeoyl-

quinic acid derivatives possessed strong DPPH free radical scavenging activity and protective activity against CCl_4-toxicity on primary cultured rat hepatocytes.[12,18] These compounds were also reported to enhance macrophage spreading and mobility.[19] Artepillin C (**5**), one of the widely studied components of Brazilian propolis, on the other hand, was reported to have strong anticancer properties.[19-22] Similarly, labdane-type diterpenes from Brazilian propolis were reported to have antimicrobial activity.[6] Likewise, flavonoids and other phenolics from Brazilian propolis possessed strong cytotoxicity toward different cancer cell lines and hepatoprotective activity.[10,23] Previously *Araucaria heterophylla*, which is rich in labdane-type diterpenes, was proposed as a possible source of Brazilian propolis by Bankova *et al.*[6] Later, we proposed that *Baccharis* species should be a possible source of Brazilian propolis on the basis of isolation of similar phenolic compounds reported from different *Baccharis* species.[10] Tazawa *et al.*, at the same time, also suggested *Baccharis* species to be a possible source of Brazilian propolis.[24] Bankova *et al.* further confirmed that **5**, **7**, **20**, **22**, **37**, **38**, dihydrocinnamic acid and 5,6,7-trihydroxy-3,4'-dimethoxyflavone of Brazilian propolis were derived from *B. dracunculifolia*.[7] While the diterpene *E/Z*-communic acid, an unique constituent of Brazilian propolis, was observed in *Araucaria angustifolia* on GC-MS analysis.[7] This finding was in accordance with their previous report that *A. heterophlla* should be the source for labdane-type diterpenes in Brazilian propolis.[6] Similarly, Park *et al.* also reported the presence of some common phenolics both in propolis of southern Brazil and the bud exudates of *B. dracunculifolia* and similar fatty components in *Hyptis divaricata* and propolis from northeastern Brazil.[8] In the present study, we for the first time detected four labdane-type diterpenes in the buds of *B. dracunculifolia*, previously isolated from Brazilian propolis,[10] *i.e.*, isocupressic acid (**10**), agathic acid (**12**), agathalic acid (**14**) and cupressic acid (**15**). These results suggested that the buds of *B. dracunculifolia* should be an important source of diterpenes found in Brazilian propolis. Caffeoylquinic acid derivatives detected in *B. dracunculifolia* and their higher intensities in the buds indicated that they should be also derived from the buds.

In conclusion, the plant source of 19 individual components of Brazilian propolis, *i.e.*, **1-3**, **6**, **10**, **12**, **14**, **15**, **17**, **19**, **21**, **26**, **27**, **28**, **31**, **32**, **34**, **39** and **41**, were established, for the first time, to be *B. dracunculifolia* and all of them except **32**, **34** and **41** were present in the buds. All these facts led us to conclude that the buds of *B. dracunculifolia* are a potent source of Brazilian propolis.

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和文抄録

ブラジル産プロポリス及び *Baccharis dracunculifolia* の葉，新芽，茎，花についてLC－MS分析を行なった。プロポリス及び *B. dracunculifolia* の水エキスは共に3,4-di-*O*-caffeoylquinic acid (**28**), 3,5-di-*O*-caffeoylquinic acid (**31**), chlorogenic acid (**32**) を含んでいた。化合物**28**及び**31**は新芽部分の水エキスが他の部分のエキスよりも強度が強く，*B. dracunculifolia* の新芽部分がこれらcaffeoylquinic acidsの重要な起源である事が示唆された。さらに，ブラジル産プロポリスの成分として報告のあるラブダン型ジテルペン，プレニル化合物，フラボノイド，桂皮酸誘導体など27化合物を *B. dracunculifolia* のメタノールエキス中に同定した。これら化合物中，24化合物が新芽部分のエキスにのみ検出された事も，*B. dracunculifolia* の新芽部分がブラジル産プロポリスの重要な起源である事を示唆している。ブラジル産プロポリスの19化合物の植物起源については，今回初めて明らかになった。

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