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Anticoagulant effect and Constituents of *Baccharis illinita*

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Chemical investigation of ethanolic extracts of the leaves and stems of *Baccharis illinita* resulted in the isolation and identification of retusin, quercetin-3, 7, 4'-trimethyl ether, aromadendrin-7-methyl ether, 3-(4-hydroxyphenyl)-2-propenoic acid (*E* and *Z*) hexacosyl ester, 3-(3, 4-dihydroxyphenyl)-2-propenoic acid (*E*) hexacosyl ester and hexacosanoyl acid acetyl ester (all new for this genus), together with apigenin-7, 4'-dimethyl ether, kaempferol-3, 7, 4'-trimethyl ether, kaempferol-7,4'-dimethyl ether, *p*-coumaric acid, hexacosanol, and stigmasta-5, 22-dien-3-ol (3β, 22*E*, 24*S*) hexadecanoyl. Their structures were assigned by comparison of the obtained spectroscopic data with those in the literature. Extracts of the flowers, roots and stems demonstrated marked anticoagulant activity, but only the extract of the stems had any significant effect on platelet aggregation.

Keywords: *Baccharis illinita*, Asteraceae, anticoagulant, flavonoids, kauranes.

Baccharis illinita DC (Asteraceae) is a small shrub, which is widely distributed in subtropical areas, especially in southern Brazil, where it is known as "erva milagrosa" or "chá ventura" and widely used in folk medicine for the treatment of several diseases. Some of the ethnomedical uses of the tea obtained from leaves and stems include use as a remedy for stomach ulcers, skin and mucosal healing, inflammation and infection. The ethnomedical use of *B. illinita* for gastric disorders has received support from the gastro-protective effects reported for a crude extract of this plant [1].

Our previous study on the flower extracts resulted in the isolation of twelve flavonoids and three kaurene diterpenoids [2]. As a continuation of our investigations into the chemical constituents of the genus *Baccharis* [3, 4], we now report the occurrence of a further eleven flavonoids, a kaurane diterpene and a few coumaric and caffeic esters of the stem and leaves of *B. illinita*. Given that this plant has high flavonoid concentrations and that phenolic substances, mainly the flavonoids, have given good results in relation to tests for vascular activity [5], we screened for anticoagulant and platelet activity.

Air-dried and powdered samples of the flowers, leaves and stems of *B. illinita* were extracted with ethanol at room temperature to give the respective extracts. Phytochemical investigation made previously of the flowers of *B. illinita* resulted in the isolation and identification of flavones **1-9**, flavanones **15-17** and kauranes **19-21** [2]. Now, after extensive chromatographic separation of the extract of leaves, we have isolated and identified flavones **8-14**, flavanones **15-16**, kaurane (**19**)**,** and *E-p*-coumaric acid hexacosyl ester (**24**). Fractionation of the stem extract afforded, besides compounds **8**, **9**, **15**, **16**, **19** and **24**, as previously identified, the flavanones **17** and **18**, *p*-coumaric acid (**22**), *Z-p*-coumaric acid hexacosyl ester (**23**), caffeic acid hexacosyl ester (**25**)**,** and stigmasta-5, 22-dien-3-ol

hexadecanoyl ester (**26**), hexacosanol (**27**) and hexacosanoic acid ethyl ester (**28**).

The above compounds were characterized by comparison of their IR, ^{1}H and ^{13}C NMR, HMBC and HMQC spectroscopic and mass spectrometric characteristics with literature data. The compounds apigenin-7, 4'-dimethyl ether (**10**) [6], retusin (**11**) [7], quercetin-3, 7, 4'-trimethyl ether (**12**) and aromadendrin-7-methyl ether (**18**) [8], kaempferol-3, 7, 4'-trimethyl ether (**13**) and kaempferol-7, 4'-dimethyl ether (**14**) [9], were confirmed by spectroscopic analysis.

The structures of *Z-p*-coumaric acid hexacosyl ester (**23**)**,** *E-p*-coumaric acid hexacosyl ester (**24**) and caffeic acid hexacosyl ester (**25**) were determined by IR, ^{1}H NMR, ^{13}C NMR, APT and HMBC spectroscopy, and MS, and by comparison with literature data [10-12]. The molecular formulae of $C_{35}H_{60}O_3$ and $C_{35}H_{60}O_4$ were assigned based on NMR spectroscopic and mass spectrometric data. The MS showed a molecular ion peak at m/z 528 $[M]^+$ for **23** and **24**, and at m/z 544 $[M]^+$ for **25**. The position of the aliphatic chain was confirmed through long-range correlations (HMBC) C-9-C-1', showing that the hexacosyl group is the alkoxy radical. Between C-7 and C-8 for compounds **23-25**, *cis* and *trans* stereochemistries were observed through the coupling constants J = 16.1 Hz and J = 12.4 Hz for a *trans* system in **24** and **25** and a *cis* system in **23**, respectively. Long chain esters of *trans* and *cis*-coumaric acid on a *n*-C_{34} and *n*-C_{30} alcohol have been isolated from *Baccharis myrsinites* [13]. The EIMS of **26** contained a $[M]^+$ peak at *m/z* 650, which corresponds to the molecular formula $C_{45}H_{78}O_2$; this is supported by the ^{1}H and ^{13}C NMR spectral data. A second ion with *m/z* 510, indicating the loss of a linear chain $C_{14}H_{28}$ from the parent molecule, relates to stigmasta-5, 22-dien-3β-ol hexadecanoyl ester. Close examination of ^{13}C NMR shifts, HMBC and HMQC confirmed this structure for **26.**

To the best of our knowledge, compounds **11**, **12**, **18,** **23**, **24**, **25**, **26**, and **28** have not been reported previously for the genus *Baccharis*, and **23-26** and **28** are new records for the family Asteraceae. The distribution of the compounds in different organs of *B. illinita* is shown in Table 1. The kaurane **19** was found in all chloroform extracts (leaves, flowers and stem), with minor amounts in the leaves. However, the kauranes **20** and **21** were only found in the flowers. Stems and leaves showed the presence of the cinnamoyl derivatives **22-25**. Phytochemical analysis showed a distribution of flavonoids with more accumulation of methoxylated flavonoids in flowers followed by the leaves and then the stems. Luteolin (**3**) and aromadendrin (**15**) are the flavonoids found in greatest concentrations in this species, but they are only present in the flowers, followed by quercetin (**9**)**,** with a high concentration in the leaves.

Table 1: Distribution of the constituents in different organs of *B. illinita.*

Compound	Flowers	Leaves	Stem
Flavonoids	**1-9, 15-17**	**8-16**	**8, 9, 15-18**
Kauranes	**19-21**	**19**	**19**
Cinnamic acid derivatives		**24**	**22-25**

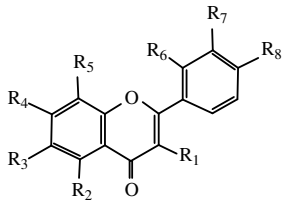
Screening tests to evaluate anticoagulant activity were carried out in relation to activated prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT). The results indicate that extracts of *B. illinita* induced significant inhibition of blood coagulation *in vitro*. A significant prolongation of APTT was observed after incubation of the plasma with 200µg/ml of the three types of extract. The extracts obtained from the leaves and stems showed the greatest coagulation inhibition (Table 2). Also, it was found that at 200 µg/ml, both extracts demonstrated a prolongation of blood coagulation in a manner similar to that of heparin. For the PT and TT tests, the results obtained demonstrate a 30% and 15% inhibition, respectively, only when utilizing a concentration of 800 µg/mL (Table 2).

The blood coagulation process involves various factors in the plasma and tissues and is regulated by extrinsic and intrinsic pathways. The substances which affect the PT act through the extrinsic pathway and may inhibit factors such as VII, V, X , II and I, whereas those which affect the APTT act through the intrinsic pathway and may inhibit all factors except VII and XIII [14]. The results obtained indicate that the extracts obtained from the leaves and stems have a greater anticoagulant effect over the APTT in comparison to the PT and TT. On the other hand, it is possible that the extracts of *B. illinita* in high concentrations enhance antithombin–III activity (coagulation inhibitor), as well as promoting the inhibition of factor X, explaining the prolongation in all tests. However, future studies are necessary to elucidate the action mechanisms.

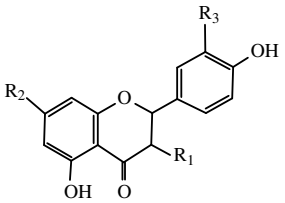
Table 2: Effect of the crude extracts from the flowers, leaves and stems of *B. illinita* on activated partial thromboplastin time (APTT), thromboplastin time (PT) and thrombin time (TT). Plasma was pre-incubated with extracts of *B. illinita* for 2 min at 37 °C before stimulation. Each value represents the mean ± SEM of 10 experiments.

Treatment		APTT (s) -	#%	PT (s) -	#%	TT (s) -	#%
Control (NaOH 0.05M)		41.5 ± 0.8	0	14.3 ± 0.1	0	16.4 ± 0.6	0
Flowers	-100µg/mL	38.0 ± 0.1	0				
	-200µg/mL	40.5 ± 0.4*	0	15.0± 0.3	4.8		
	-400µg/mL	45.0 ± 0.5*	8.4	15.4± 0.2	7.6	16.0± 0.3	0
	-800µg/mL	64.6 ± 0.7*	55.6	16.2± 0.2	13.2	20.1± 0.1*	22.5
Leaves	-100µg/mL	51.6 ± 0.8	24.3				
	-200µg/mL	56.3 ± 0.9 *	35.6	16.2 ± 0.5	13.2		
	-400µg/mL	75.7 ± 1.8 *	82.4	17.2 ± 0.7	20.2	17.3 ± 0.4	5.4
	-800µg/mL	141.2 ± 6.0*	>100	19.1 ± 0.6 *	33.5	18.9 ± 0.3*	15.2
Stems	-100µg/mL	49.4 ± 1.5	19.0				
	200µg/mL	53.7 ± 0.6*	29.3	16.1 ± 0.3	12.5		
	-400µg/mL	73.8 ± 0.7*	77.8	16.2 ± 0.5	13.2	16.1 ± 0.3	0
	-800µg/mL	123.9 ± 5.7*	>100	18.5 ± 0.2*	29.3	19.0 ± 0.8*	15.8
Heparin 0.25UI/mL		57.7 ± 0.6 *	39.0	19.9 ± 0.6*	39.1	33.9 ± 3.0*	>100

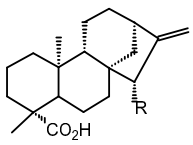
* Statistics: Student-Newman-Keuls test; $p < 0.05$*; n=10; $p < 0.05$; # % of inhibition

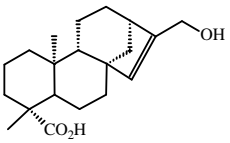


	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	H	OMe	OMe	OMe	OMe	H	OMe	OMe
2	H	OMe	OMe	OMe	OMe	H	H	OMe
3	H	OH	H	OH	H	H	OH	OH
4	OH	OH	H	OH	H	H	H	OH
5	H	OH	H	OH	H	OMe	H	H
6	OMe	OH	H	OH	H	H	OH	OH
7	H	OH	H	OH	H	H	OMe	OH
8	H	OH	H	OH	H	H	H	OH
9	OH	OH	H	OH	H	H	OH	OH
10	H	OH	H	OMe	H	H	H	OMe
11	OMe	OH	H	OMe	H	H	OMe	OMe
12	OMe	OH	H	OMe	H	H	OH	OMe
13	OMe	OH	H	OMe	H	H	H	OMe
14	OH	OH	H	OMe	H	H	H	OMe



	R_1	R_2	R_3
15	OH	OH	H
16	H	OH	H
17	OH	OH	OH
18	OH	OMe	H





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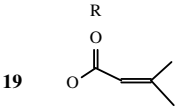
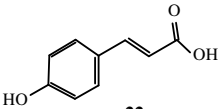


19

20 OH



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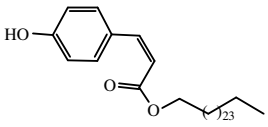

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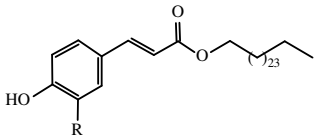
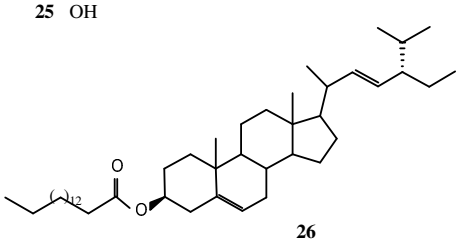


R

24 H

25 OH



26

In order to evaluate the effects of *B. illinita* extracts on platelet aggregation, the extracts were pre-incubated at a concentration of 800 µg/mL and then stimulated with 6 µM-ADP. The results obtained do not demonstrate significant inhibition of platelet aggregation in the presence of extracts originating from the flowers and leaves.

On the other hand, the extract obtained from the stems showed a slight anti-aggregation activity (inhibition of 17%) in relation to the control. In conclusion, the results obtained demonstrate that *B. illinita* shows anticoagulant action preferentially by inhibition of the intrinsic pathway of blood coagulation.

Experimental

Chromatography column CC: silica gel (Merck) 70–120 mesh, 230–400 mesh and Sephadex L-20; IR spectra were recorded on a FT Perkin Elmer 16 PC spectrophotometer as KBr disks; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra in $CDCl_3$, acetone-d_6 and CD_3OD were recorded on a JEOL Eclipse+ 400 spectrometer.

Reagents: The reagents PT and APTT tests were purchased from Wiener Lab, Argentina. The thrombin test (TT) reagent was obtained from Organon Technika (Durham, NC, USA) and adenosine diphosphate (ADP) from Chronolog Corporation, USA. The study included 10 volunteers, who gave informed consent before participating in this study.

Plant material: Aerial parts of *Baccharis illinita* DC (Asteraceae) were collected in Serra do Falcão, Minas Gerais, and identified by botanist José Badini. A voucher specimen (OUPR N. 3450) was deposited in the Herbarium of the Universidade Federal de Ouro Preto, Minas Gerais, Brazil.

Extraction and isolation procedure: The stems, leaves and flowers were separated, air-dried, powdered and extracted with 96% ethanol at room temperature for 15 days. The extracts were filtered and concentrated at reduced pressure, to give the crude extracts for each part of the *B. illinita* plant. Crude extracts of the stem and leaves were partitioned between $CHCl_3/H_2O$ to give $CHCl_3$ and aqueous soluble fractions.

$CHCl_3$ soluble fraction of the leaves: This fraction (10 g) was subjected to column chromatography using silica gel (70-230 mesh) and eluting with n-hexane-EtOAc and EtOH-EtOAc gradient system to yield 49 fractions. Fractions 14-17 and 18-21 were subjected to Sephadex column chromatography eluting with $CHCl_3$-MeOH (7:3) to give diterpene **19** (86 mg), *p*-coumaric acid (**22**) (36mg) and a *p*-coumaric acid hexacosyl ester (**24**) (20 mg), respectively.

Aqueous soluble fraction of the leaves: This fraction (8 g) was chromatographed on silica gel using the *n*-hexane-EtOAc/ EtOH-EtOAc gradient system to obtain 57 fractions. The combined fractions 12-15 were recrystallized from acetone to give **16** (149 mg). Fraction 16, after recrystallization from acetone, afforded **15** (62 mg). The combined mother liquor of frs 12-15 and 16 was subjected to flash chromatography over silica gel (230-400 mesh) using EtOAc-*n*-hexane (1:1) as the eluent to give frs 1A–12A. Frs 2A–4A were recrystallized from acetone to yield apigenin-7, 4'-dimethyl ether (**10**) (14 mg). Further flash chromatography of frs 5A–10A, eluting with EtOAc-*n*-hexane (1:1), afforded retusin (**11**) (19 mg) and a mixture of flavonoids (**12-14**). These mixtures were further purified by flash chromatography to yield quercetin-3, 7, 4'-trimethyl ether (**12**) (8 mg), kaempferol-3, 7, 4'-trimethyl ether (**13**) (5 mg) and kaempferol-7, 4'-dimethyl ether (**14**) (28 mg). The combined fractions 19–20 and 21–24 were purified by recrystallization from acetone to give **9** (325 mg) and **8** (90 mg), respectively.

$CHCl_3$ soluble fraction of the stems: This fraction (10 g) was subjected to column chromatography over silica gel (70-230 mesh), eluting with a gradient of *n*-hexane-EtOAc/ EtOAc-EtOH to afford 42 fractions. Fractions 1 – 2 and 5 were purified by recrystallization from $CHCl_3$, yielding stigmasta-5, 22-dien-3-ol (3β, 22*E*, 24*S*) hexadecanoyl (**26**) (27 mg) and compound **19** (195 mg), respectively. Fraction 20 was rechromatographed in a flash column on silica gel (230 – 400 mesh) using EtOAc-*n*-hexane (15:85 and 1:1) as the eluent to give frs 1A – 39A. From frs 5A – 7A hexacosyl *p*-coumarate (Z) (**23**) (14 mg), was obtained in a mixture with hexacosanol (**27**) and hexacosanoyl acid acetyl ester (**28**). Frs 9A –15A yielded hexacosyl *p*-coumarate (*E*) (**24**) (22 mg). Fr 26A was rechromatographed on silica gel in a flash column using EtOAc-*n*-hexane (1:4) as the eluent to give frs 1B – 22B. Frs 9B – 16B yielded 3-(3, 4-dihydroxyphenyl)-2-propenoic acid (*E*) (**25**) (19 mg).

Aqueous soluble fraction of the stems: This fraction (5 g) was chromatographed on silica gel using gradient elution with *n*-hexane-EtOAc/ EtOH-EtOAc to give 46 fractions. Recrystallization from acetone of fractions 2, 4-5, 6 and 8-9 afforded **16** (9 mg), **8** (18 mg), **15** (16 mg) and **9** (33 mg), respectively. The combined fractions 10-11 and mother liquor of the fractions 8-9 were subjected to chromatography over silica gel to give the flavonoids **9** and **17** (34 mg) and aromadendrin-7-methyl ether (**18**) (8 mg).

Measurement of Blood Coagulation: Human blood was obtained from 10 healthy donors without a history of either bleeding or thrombosis. Nine parts of blood collected by venipuncture were drawn into one part of 3.8% trisodium citrate. Blood was centrifuged for 10 min at 900 *g*, and the plasma stored at -20 ºC until use. Activated prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were evaluated according to Triplett [15]. Plasma was pre-incubated with different concentrations of extracts or a vehicle (0.05 M NaOH) for 2 min at 37 ºC and coagulation time was measured using a Net Lab Digital Coagulation System.

Isolation of platelets: Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at room temperature for 7 min at 400 *g*. Platelets were adjusted to 3.0 x 10^8 cell/mL with sterile saline solution.

Measurement of platelet aggregation (PA) - induced by ADP: PA was determined by the turbidimetric method [16] using a Chronolog Corp., USA aggregometer. Aliquots (400 µL) of a platelet suspension were transferred into small cuvettes and stirred at a constant speed of 180 *g* at 37 ºC. The platelets were pre-incubated with different extracts of either *B. illinita* or a vehicle (0.05 M NaOH) for 5 min at 37 ºC, prior to the addition of 6 µM-ADP. The extent of aggregation (%) was recorded continually for 5 min after addition of the agonist.

***Statistical Analysis*:** Data were expressed as mean ± SEM. The Student-Newman-Keuls test was employed to determine whether there were differences between the groups. Differences were considered significant when the probability was $p < 0.05$. The statistical program Instat-2 was utilized.

References

[1] Baggio CH, Freitas CS, Rieck L, Marques MCA. (**2003**) Gastroprotective effects of a crude extract of *Baccharis illinita* DC in rats. *Pharmacological Research*, ***47***, 93-98.

[2] Verdi LG, Brighente IMC, Schripsema J, Braz Filho R, Pizzolatti MG. (**2004**) Kaurene diterpenes and flavonoids from *Baccharis illinita* flowers. *Biochemical Systematics and Ecology*, ***32***, 837-840.

[3] Moreira FPM, Coutinho V, Montanher ABP, Caro MSB, Brighente IMC, Monache FD, Pizzolatti MG. (**2003**) Flavonóides e triterpenos de *Baccharis pseudotenuifolia* – bioatividade sobre. *Artemia salina*. *Química Nova*, ***26***, 309-311.

[4] Moreira FPM, Branco A, Pizzolatti MG, Morais AA, Monache FD. (**2003**) Acid triterpenes and flavonoids from *Baccharis ligustrina* (Asteraceae). *Biochemical Systematics and Ecology*, ***31***, 319-321.

[5] Harborne JB, Williams CA. (**2000**) Advances in flavonoid research since 1992. *Phytochemistry*, ***55***, 481-504.

[6] Yang HO, Suh DY, Ham BH. (**1995**) Isolation and characterization of platelet – activating factor receptor binding antagonists from *Biota orientalis*. *Planta Medica*, ***61***, 37-40.

[7] Sy LK, Brown GD. (**1998**) Three sesquiterpenes from *Artemisia annua*. *Phytochemistry*, ***48***, 1207-1211.

[8] Agrawal PK. (**1989**) Studies in organic chemistry 39: Carbon-13 NMR of flavonoids. Elsevier, New York.

[9] Rossi MH, Yoshida M, Maia JGS. (**1997**) Neolignans, styrylpyrones and flavonoids from an *Aniba* species. *Phytochemistry*, ***45***, 1263-1269.

[10] Hashidoko Y, Tahara S, Mizutani J. (**1992**) Long chain alkyl esters of 4'-hydroxy cinnamic acids from leaves of *Rosa rugosa*. *Phytochemistry*, ***31***, 3282-3283.

[11] Saha MM, Mallik UK, Mallik AK. (**1991**) A chromenoflavanone and two caffeic esters from *Pongamia glabra*. *Phytochemistry*, ***30***, 3834-3836.

[12] Chatterjee A, Saha SK, Bhattacharya S. (**1980**) Hexacosyl *p*-coumaric, a new phenolic ester from Dikamali Gum. *Indian Journal of Chemistry*, ***19B***, 421-422.

[13] Li CJ, Ahmed AA, Arias ADC, Mabry TJ. (**1997**) Clerodane diterpenoids long chain esters of coumaric acid and other compounds from *Baccharis myrsinites*. *Phytochemistry*, ***45***, 571-574.

[14] Colman RW, Hirsh J, Marder VJ, Salzman EL. (**1998**) Haemostasis and thrombosis: basic principles and clinical practice. 3th ed., J. B. Lippincott Company, Philadelphia, 1-18.

[15] Triplett DA, Harms CS, Koepke JA. (**1978)** The effect of heparin on the activated partial thromboplastin time. *American Society of Clinical Pathology*, ***70***, 556-559.

[16] Born GVC, Cross MJ. (**1963**) Aggregation of blood platelets. *Journal Physiology-London*, ***168***, 178-&.