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Phenolic compounds and triterpene identified from *Monanthotaxis pilosa* Baillon

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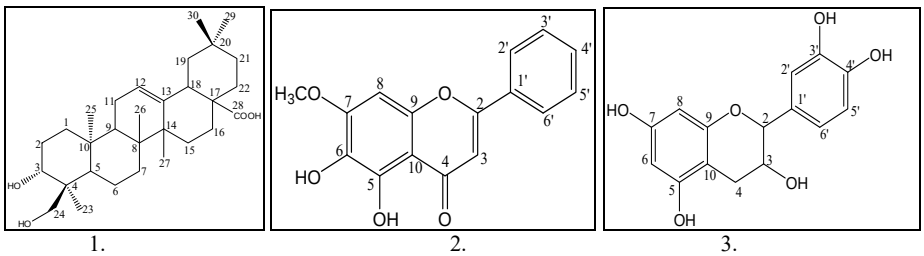
Abstract
The aim of this work was to evaluate and isolate chemical constituents of *Monanthotaxis pilosa*, an endemic medicinal plant from Madagascar. Fourteen compounds were identified. Three of them were isolated from ethyl acetate leaves extract on silica column and identified to epihederagenine 1, negletein 2 and epicatechin 3 by spectrometric techniques (NMR) and DRX. An high-performance liquid chromatography/mass spectrometry method was developed to investigate the presence of phenolic compounds in the studied samples; coumaric acid, chlorogenic acid, ferulic acid, hyperoside, isoquercitrin, rutin, quercitrin, quercetol, kaempferol, apigenine and myricetol were identified. Total flavonoid content was determined by a spectrophotometric method using $AlCl_3$ reagent. The leaves of *Monanthotaxis pilosa* can be considered an important source of flavonoids, especially of quercitrin.

Keywords: *Monanthotaxis pilosa* Baill, flavonoids, triterpene, phenolic acids, DRX, HPLC, SM, RMN

1. Introduction
The *Monanthotaxis* genus of the Annonaceae family includes more than 55 species of climbing shrubs, woody vines and evergreen, the leaves alternate, simple and entire[1], distributed mainly in tropical regions of the Old Continent.
In Madagascar, we found 11 genera with eighteen species endemics are encountered [1, 2]. Chemical investigation of *Monanthotaxis* species has led to the isolation of alkaloids [3, 4, 5], terpenoids [7] and polyphenolic compounds such polymethoxy flavones [6]. *Monanthotaxis pilosa* (Baill.) has not previously been investigated phytochemically.
Monanthotaxis pilosa syn. *Popowia pilosa* is a wild plant [8] and the leaves and bark are used in traditional medicine to treat fever with flu-like symptoms and neurological signs, painful menstruation, and then to cure the disease-related liver problem called "Ambo ".
In this study, crude ethanolic extracts of leaves, bark of *Monanthotaxis pilosa* Baillon, species used in traditional medicine in Madagascar, were analyzed. Three compounds were isolated from AcOEt leaves extract by column chromatographic separation at low pressure and identified by spectrometric techniques (NMR 1D, 2D) or and DRX to epihederagenine 1, negletein 2 and epicatechin 3. Eleven phenolic compounds were identified by mass spectrometry coupled with liquid chromatography high performance (HPLC / MS).



1. 2. 3.

2. Materials and methods
2.1 Plant material
The leaves and bark of *M. pilosa* were harvested in Joffreville, 20 km from the town of Antsiranana Madagascar in December 2013 (sample Voucher N° DSM 423). The species was identified at the Botanical and Zoological Park Tsimbazaza Antananarivo, Madagascar and an

herbarium were deposited in the "Natural Substance and Organic Chemistry Laboratory of Chemistry (LCSN / COB).

2.2 General experimental procedures

2.2.1 Extraction and isolation

400 g powder of *Monanthotaxis pilosa* leaves were extracted successively with n-hexane (n-Hex), dichloromethane (DCM), ethyl acetate (AcOEt) and n-butanol (n-BuOH). The preliminary study has shown that the AcOEt extract exhibited the highest antioxidant activity. Therefore, our investigation has been focused on this extract.

Sixty grams of silica gel (60 F254; 70-230 Mesh) were prepared in cyclohexan and packed in column (length 100 cm, internal diameter 3.5 cm). Two grams of the AcOEt extract was subjected onto the column chromatography and eluted with a gradient cyclohexan - AcOEt (100:0; 80:20; 70:30; 60:40; 20:80); 135 aliquots of 10 ml each were collected and analysed with thin layer chromatography (TLC), using precoated silica gel (60 F254) plates aluminium base (Merck) of 0.2 mm thickness. Five microliters of aliquots were deposited on TLC plates and eluted with a mobile phase cyclohexan - AcOEt. The plates were dried and separated compounds were detected under UV lamp (254/365 nm) or by spraying with freshly prepared sulphuric acid spray reagent and then heated at 110 °C for 10 min. The spots exhibiting the same R*f* on TLC were combined. The fractions 65 to 80 eluted with cyclohexan – AcOEt (80:20) were combined and precipitated with MeOH to give compound 1 (white powder, 15 mg). The fractions 99-120 [20.6 mg, cyclohexan – AcOEt (60:40)] were purified by ethylic ether to obtain compounds 2 (R_f =0.15, yellow crystals gold, 20 mg). The fractions 120-130 [20.6 mg, cyclohexan – AcOEt (20:80)] were purified by ethylic ether to obtain compounds 3 (yellow powder, 25 mg). The structure of isolated compound was elucidated by spectral data (1D- and 2D-NMR, MS) and or DRX.

2.2.2 HPLC / MS analysis

One g of the powder of plant material (leaves, bark) was extracted with 10 ml of ethanol (EtOH / H_2O - 70/30) for 30 min with a reflux device at 95 °C. After filtration through Whatman No. 1 filter paper, the filtrate was cooled and centrifuged (4000 rpm, 30 min) and then stored at 4 °C until analysis [9].

Apparatus. The experiment was carried out using an Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser, binary gradient pump -, column thermostat, autosampler and UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18 100 x 3.0 mm i.d., 3.5 µm particle); the work temperature was 48 °C. The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using Chem Station and Data Analysis software from Agilent, USA.

2.2.2.1 Chromatographic conditions of methylated flavone analysis

The detection of the *methylated flavone* was performed on MS/MS mode MRM or SRM. The mobile phase was a binary gradient prepared from methanol and solution of acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 45% methanol and ending at 50% methanol, for 8 minutes. The flow rate was 0.9 mL min^{-1} and the injection volume was 5 µL.

2.2.2.2 Chromatographic conditions of other polyphenol analysis

The detection of the other polyphenol was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min, then at 370 nm.

The mobile phase was a binary gradient prepared from methanol and solution of acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 min; isocratic elution followed for the next 3 min with 42% methanol. The flow rate was 1 mL min^{-1} and the injection volume was 5 µL.

2.2.2.3 Identification and quantitative determinations of polyphenols

The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library. Later, the MS traces/spectra of the analysed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral mach. The UV trace was used for quantification of identified compounds from MS detection. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5–50 µg mL^{-1} range with good linearity (R^2 > 0.999) for a five point plot were used to determine the concentration of polyphenols in plant samples [10, 11, 12].

2.2.2.4 Identification and quantitative determinations of methylated flavone

Identification of the methylated flavone was based on accurate mass measurements of the pseudomolecular $[M-H]^-$. Methylated flavones in ethanolic extract were identified by comparing the retention times, absorption spectra (200–600 nm) and mass spectra of unknown peaks with the available reference standards (jaceosidin 2,9 min, hispiduline 4,2 min, eupalitin 7,05 min, eupatorin 7,6 min, acacétine 8,05 min and casticine 9,8 min) and the compounds were numbered by the order of elution [13].

2.2.3 Total flavonoid content by a spectrophotometric method

The concentration of flavonoids in plant extracts was determined using spectrophotometric method. Then, 1 g of powdered vegetal product was extracted with 20 mL of ethanol 50% (v/v) for 30 min on a water bath at 95 °C. After cooling, the solution was filtered and completed at 25 mL in a volumetric flask with the same solvent. Then, 10 mL of this solution is diluted in a 25 mL volumetric flask with the same solvent, stirring 2 to 3 min and let standing for 10 min. At 5 mL of this solution was added 5 ml CH_3COONa 10 g / L, 3 ml of $AlCl_3$ 25 g / L and completed in a volumetric flask to 25 mL with distilled water. The absorbance was determined at 430 nm, using a UV-VIS JASCO V-530 (Tokyo, Japan) spectrophotometer and a mixture of solution B (5 mL), distilled water (8 mL) and methanol (12 mL) as compensation liquid, after 15 min storing in the darkness.

The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of dry material) [14, 15].

3. Results and discussions

3.1 Isolated compounds

Structures were assigned by analysis of the ^{1}H, ^{13}C and 2D NMR spectra, DRX and by comparison with literature values. By concerted use of one and two dimensional NMR spectroscopy, compound 1 was exhibited the same chemical shift (^{13}C) as hederagenine [16] except carbon in position 3. Therefore, for compound 1 the chemical shift for C-3 (δ_C 70.7 ppm) is too low. Thus compound 1 is identified to 3α, 24-hydroxyolean-12-en-28-oic acid named 3-epihederagenine. By concerted use of one and two dimensional NMR spectroscopy, compound 2 was identified as catechin [17], compound 3 as negletein [18]. Therefore, the structure of compound 3 is confirmed by DRX method.

Compound 1: white powder (15 mg) from (Hex / EtOAc, 80/20, v / v)

^{1}H NMR (600 MHz, DMSO- d6) δ ppm : 1.44, 0.81 (2H, m, H-1) ; 1.48 (2H, H-2) ; 3,47 (1H, H-3) ; 1.12 (1H, H-5) ; 1.18, 1.35 (2H, m, H-6) ; 1.43, 1.15 (2H, m, H-7) ; 1.48 (1H, H-9) ; 1.47, 1.90 (2H, m, H-11) ; 5.15 (1H, H-12) ; 0.94, 1.48 (2H, m, H-15) ; 1.47, 1.90 (2H, m, H-16) ; 2.75 (1H, H-18) ; 1.03, 1.60 (2H, m, H-19) ; 1.27, 1.27 (2H, m, H-21) ; 1.60, 1.41 (2H, m, H-22) ; 3.08, 3.33 (2H, d, H-24) ; 0.53 (3H, s, H-23) ; 0.86 (3H, s, H-25) ; 0.72 (3H, s, H-26) ; 1.10 (3H, s, H-27) ; 0.86 (3H, s, H-29) ; 0.83 (3H, s, H-30).

^{13}C NMR (125 MHz, DMSO-d6) δ ppm: 38.38 (C-1) ; 26.96 (C-2) ; 70.71 (C-3) ; 42.31 (C-4) ; 46.83 (C-5) ; 17.94 (C-6) ; 32.55 (C-7) ; 39.21 (C-8) ; 47.55 (C-9) ; 36.75 (C-10) ; 23.34 (C-11) ; 122.04 (C-12) ; 144.34 (C-13) ; 41.81 (C-14) ; 27.65 (C-15) ; 23.03 (C-16) ; 45.92 (C-17) ; 41.27 (C-18) ; 46.14 (C-19) ; 30.86 (C-20) ; 33.77 (C-21) ; 32.44 (C-22) ; 64.83 (C-24) ; 13.10 (C-23) ; 15.99 (C-25) ; 17.35 (C-26) ; 26.11 (C-27) ; 176.80 (C-28) ; 33.30 (C-30) ; 23.83 (C-29).

Compound 2: yellow crystals - gold (20 mg) from (Hex / EtOAc, 60/40, v / v)

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8,58 (2H, d, J); 8,21 (2H, dd,); 8,18 (2H, m); 7,39
(1H, s): 7,38 (1H, s), 4,4 (3H, s)

^{13}C NMR (100 MHz, CDCl3) δ ppm: 183,08 (C = O); 164,6 (Cq); 154,3 (Cq); 150,9 (Cq); 132 (CH); 131,9 (Cq); 130,3 (CH); 128,7 (CH); 126,4 (CH); 106,7 (Cq); 56.4 (CH3). The

Compound 3: yellow powder (30 mg) from (Hex / EtOAc, 20/80, v / v)

^{1}H NMR (600 MHz, DMSO- d6) δ ppm : 4.73 (1H, s, H-2) ; 4.01 (1H, s, H-3) ; 2.46 et 2.65 (2H, dd, H-4) ; 5.89 (1H, d, H-6) ; 5.72 (1H, d, H-8) ; 6.88 (1H, d, H-2') ; 6.66 (1H, d, H-5') ; 6.67 (1H, dd, H-6').

^{13}C NMR (120 MHz, DMSO-d6) δ ppm : 78.50 (C-2) ; 65.35 (C-3) ; 29.43 (C-4) ; 156.98 (C-5) ; 95.51 (C-6) ; 156.67 (C-7) ; 94.53 (C-8) ; 156.22 (C-9) ; 98.95 (C-10) ; 131.06 (C-1') ; 115.33 (C-2') ; 144.88 (C-3') ; 144.94 (C-4') ; 115.20 (C-5') ; 118.40 (C-6').

3.2 High-performance liquid chromatography/mass spectrometry

A method of coupling HPLC with UV and MS was optimised for the separation and identification of phenolic acids, flavonoid glycosides, flavonoid aglycones and methylated flavone (phenolic compounds) in the ethanolic 70% (v/v) extracts of *M. pilose* [19]. In this study, 24 standard phenolic compounds (Table 1) have been investigated in ethanolic extracts. Using the chromatographic conditions described above, the polyphenols were eluted in less than 35 minutes (table 1). Four polyphenols of standard phenolic compounds cannot be quantified in current chromatographic conditions due overlapping (caftaric acid with gentisic acid and caffeic acid with chlorogenic acid). However, all four compounds can be selectively identified in MS detection (qualitative analysis) based on differences between their molecular mass and MS spectra.

Table 1. Retentions times (min) for standard polyphenolic compounds.

Peak no.	Polyphenolic compounds	m/z	RT± SD	Peak no.	Polyphenolic compounds	m/z	RT± SD
1	Caftaric acid*	311	2.10± 0.06	13	Quercitrin	447	23.00 ± 0.13
2	Gentisic acid*	153	2.15± 0.07	14	Quercetol	301	26.80 ± 0.15
3	Caffeic acid*	179	5.60± 0.04	15	Patuletin	331	28.70 ± 0.12
4	Chlorogenic acid*	353	5.62± 0.05	16	Luteolin	285	29.10 ± 0.19
5	p-Coumaric acid	163	8.7± 0.08	17	Kaempferol	285	31.60 ± 0.17
6	Ferulic acid	193	12.2 ± 0.10	18	Apigenin	279	33.10 ± 0.15
7	Sinapic acid	223	14.3 ± 0.10	19	Jaceosidin**	330.3	2.9
8	Hyperoside	463	18.60 ± 0.12	2.0	Hispidulin**	300.2	4.2
9	Isoquercitrin	463	19.60 ± 0.10	21	Eupalitin**	344.3	7.05
10	Rutoside	609	20.20 ± 0.15	22	Eupatorin**	344.3	7.6
11	Myricetin	317	20.70 ± 0.06	23	Casticin**	374.3	8.05
12	Fisetin	285	22.60 ± 0.15	24	Acacetin**	284.3	9.8

* overlapping in UV detection, only qualitative analysis possible using MS detection SD, standard deviation
**methylated flavone

The simultaneous analysis of different classes of polyphenols in ethanolic extracts was performed by a single column pass, and the separation of all examined compounds was carried out in 35 min. After analysis, fifteen of them were identified, nine in *M. pilosa* leaves (three cinnamic acids derivatives: chlorogenic acid, coumaric acid and ferulic acid; four flavonoid glycosides: hyperoside, rutin, isoquercitrin and quercitrin; two flavonols: quercetin and kaempferol) and six also in *M. pilosa* bark (three flavonoid glycosides: hyperoside, isoquercitrin and quercitrin; one flavone: apigenin; two flavonols: quercetin and myricetol) by comparing retention times (RTs), UV and MS data with those of the reference standards [12]. The chromatograms of phenolic acids and flavonoids (glycosides, aglycones and methylated flavones) for two ethanolic extracts (leaves and bark) are presented in Figures 2, 3, 4 and 5.

The amounts of identified polyphenolic compounds in the analyzed samples are reported respectively in Table 2 and Table 3.

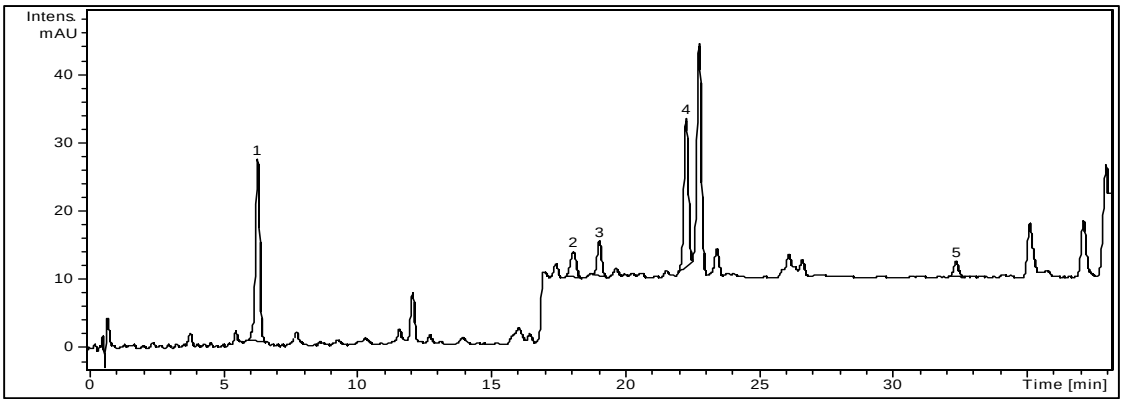


Fig 2: Chromatographic profile of the ethanolic extract of leaves of *Monanthotaxis pilosa.* Phenolic compounds identified : chlorogenic acid (1); hyperoside (2); isoquercetin (3); rutoside ; quercitrin (4); quercetol ; kaempferol (5).

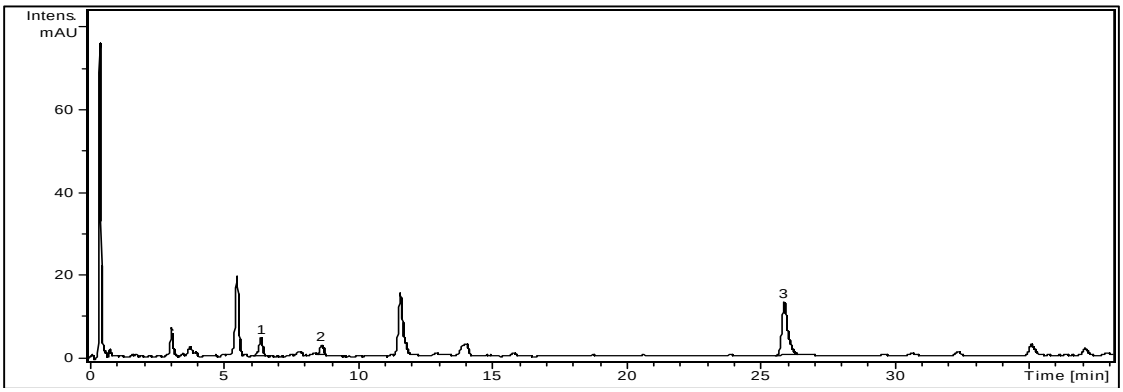


Fig 3: Chromatographic profile of the ethanolic extract of the leaves of *Monanthotaxis pilosa* for identification of methylated flavone compounds: chlorogenic acid (1); coumaric acid (2); ferulic acid ; quercetol.

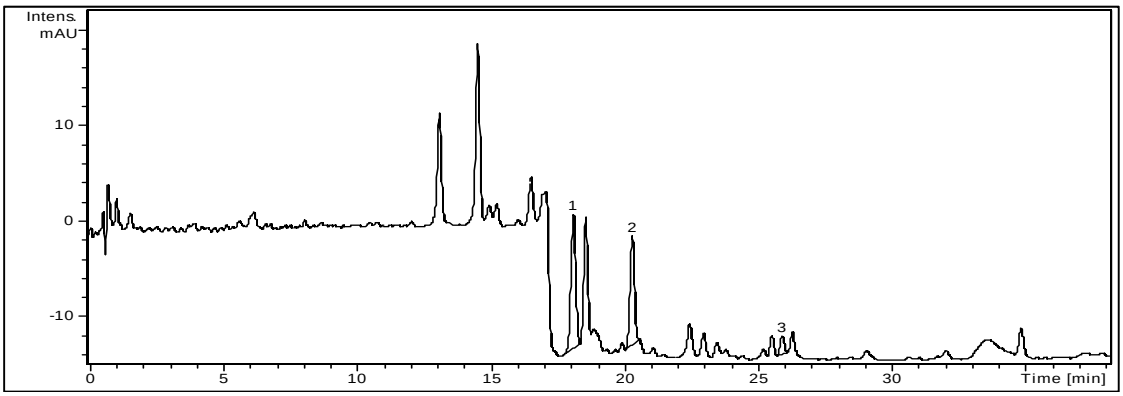


Fig 4: Chromatographic profile of the ethanolic extract of bark *Monanthotaxis pilosa* for identification of phenolic compounds: hyperoside (1); isoquercitrin (2); miricetol; quercetol (3).

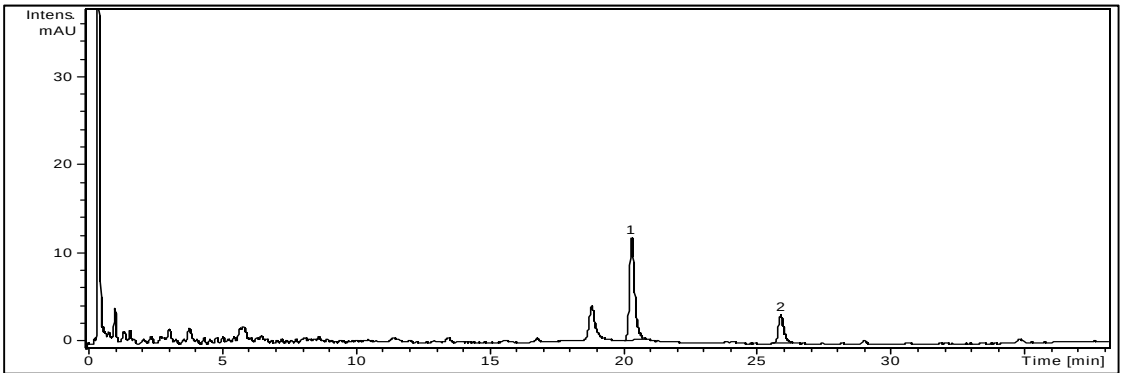


Fig 5: Chromatographic profile of the ethanolic extract of bark of *Monanthotaxis pilosa* for identification of methylated flavone compounds: miricetol (1); quercetol (3).

Table 2: Products identified and their concentrations of the phenolic compounds contained from the leaves and bark of *Monanthotaxis pilosa*.

Polyphenolic compounds	m/z value	RT ± SD (min)	Concentrations in leaves (µg/100 g)	Concentrations in bark (µg/100g)
Chlorogenic acid	353	6.43 ± 0.05	23.880	-
Hyperoside	463	19.32 ± 0.12	6.208	20.099
Isoquercétin	463	20.29 ± 0.12	9.597	-
Rutin	609	20.76 ± 0.15	-	-
Myricetin	317	21.13±0.12	-	11.069
Quercitrine	447	23.64±0.13	50.096	-
Quercetin	301	27.55 ± 0.15	-	1.330
Kaempferol	285	32.48 ± 0.17	2.207	-
Apigenin	279	39.45±0.15	-	-

Note: –, not found.

Table 3: Products identified and their concentrations for methylated flavone compounds contained in the extract from the leaves and bark of *Monanthotaxis pilosa*.

Polyphenolic compounds	m/z value	RT ± SD (min)	Concentrations in leaves (µg/100g)	Concentrations in bark (µg/100g)
Chlorogenic acid	353	6.43 ± 0.05	3.573	-
Acid β-Coumaric	163	9.48±0.08	1.524	-
Ferulic acid	193	12.8±0.10	-	-
Myricetin	317	21.13±0.12	-	11.681
Quercetin	301	27.55 ± 0.15	11.735	2.431

Note: –, not found.

In *M. pilosa* ethanolic leaves extract, three types of phenolic compounds which are cinnamic acid derivatives, flavonoid glycosides and flavonoid aglycones were identified. Quantitative results showed that quercitrin (50.096 µg/100 g) has the highest concentration value over other phenolic compounds: Chlorogenic acid 23.880 µg/100 g**,** isoquercitrin 9.597 µg/100 g, hyperoside 6.208 µg/100 g and kaempferol (2.207 µg/100 g) were identified and quantified in the extract; one cinnamic acid derivative (ferulic acid) and one flavonoid glycosides (rutin), were identified but the concentrations were too low to be quantified (Table 2). No methylated flavone was detected in ethanolic leaves extract (table 3) but in this test other phenolic compounds no detected above were identified and quantified quercetin 11.735 µg/100 g and coumaric acid (1.524 µg/100 g).

In *M. pilosa*, ethanolic bark extract, isoquercitrin, quercitrin, and apigenin were identified, but the concentrations were too low to be quantified. No methylated flavone was detected (table 3). In these samples, three flavonoids were identified and quantified: hyperoside (flavonoid glycoside) was the compound found in the highest amount (20.099 µg/100 g), followed by myricetin 11.681 µg/100 g and quercetin 2.431 µg/100 g two flavonoid aglycones quantified in the analysed of methylated flavone (table 3). Ethanolic leaves extracts of *M. pilosa* are rich in flavonoid glycosides (hyperoside, rutin, isoquercitrin and quercitrin), whereas those values were lower in stem bark extract.

3.3 Total flavonoid content by a spectrophotometric method

The total flavonoid content of the plant extracts was expressed in milligrams of rutin equivalent per gram of dry material (mg rutin/g dry material), as shown in Table 4. Total flavonoid content was higher in leaves than in stem bark ethanolic extracts.

Table 4: Flavonoid content of *M. pilosa*.

Sample	Absorbance (nm)	Flavonoid content mg rutin / g dry material
Leaves of *M. pilosa*	0,7140	3,72
Bark of *M. pilosa*	0,4823	2,47

4. Conclusion

This work has demonstrated those leaves and bark of *Monanthotaxis pilosa* Baillon are rich in phenolic compounds: phenolic acids, flavonoids such the other species of *Monanthotaxis* genus.

To our knowledge, this is the first report that shows the chemical analysis of *Monanthotaxis pilosa* leaves and bark ethanolic extracts. Some phenolic compounds were identified and quantified by HPLC/MS, such as rutin, isoquercitrin, hyperoside, quercitrin, quercetin, apigenin and luteolin. Three compounds: two flavonoids (epicatechin, negletein) and one triterpene (epihederagenine) were isolate from the leaves and identified by NMR or and DRX.

Further research is necessary in order to know all the active principles and their pharmacological properties.

5. Acknowledgements

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