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Biodiversity and traditional medicinal plants from Madagascar: Phytochemical evaluation of *Brachylaena ramiflora* (DC.) Humbert decoctions and infusions

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Summary

Madagascar is characterized by one of the highest rates of endemism and biodiversity in the world. *Brachylaena* preparations are extensively used in Malagasy folk medicine for treating gastrointestinal diseases and blenorrhagia. The aim of this study was a preliminary phytochemical fingerprint of *Brachylaena ramiflora* leaves infusions and bark decoctions, in order to characterize this species as source of biologically active compounds and their relative antioxidant activity by high-performance liquid chromatography-diode array detector. Sixteen and twenty-three biomarkers (molecules with health properties selected for their demonstrated positive role on human organism) were identified in *B. ramiflora* leaf infusions and bark decoctions, respectively: the main compounds identified in the infusions were quinic acid (334.55±0.99 mg/100 g_{FW}), chlorogenic acid (208.27± 7.74 mg/100 g_{FW}), and g-terpinene (144.19±1.00 mg/100 g_{FW}), while the major components in the decoctions were castalagin (2002.64± 13.96 mg/100 g_{FW}), citric acid (1171.81±1.05 mg/100 g_{FW}), and chlorogenic acid (646.44±2.31 mg/100 g_{FW}). *B. ramiflora* could be considered as a promising source of natural antioxidants that may provide health-benefits. The development of pharmaceuticals based on a sustainable exploitation of wild medicinal plants or their cultivation by local villagers could offer a number of benefits to a wide range of people as an alternative source of income and a natural and accessible health remedy.

Keywords: medicinal tree-species, antioxidant activity, phytochemical fingerprint, ethnobotany, endemism.

List of abbreviations: 1,2-phenylenediamine dihydrochloride: OPDA; ascorbic acid: AA; dehydroascorbic acid: DHAA; Botanical and Zoological Park of Tsimbazaza: BZPT; normal atmosphere: N.A.; relative humidity: R.H.; high-performance liquid chromatography: HPLC; diode array detector: DAD; total polyphenolic content: TPC; gallic acid equivalents: GAE; fresh weight: FW; total anthocyanin content: TAC; cyanidin-3-O-glucoside: C3G; ferric reducing antioxidant power: FRAP; polytetrafluoroethylene: PTFE; fluorophore 3-(1,2-dihydroxyethyl)furo(3,4-b)quinoxalina-1-one: DFQ; total bioactive compound content: TBCC; Pearson's correlation coefficients: R

Introduction

The World Health Organization estimates that nearly 80% of the population in developing countries depends mainly on traditional medicine for the treatment of ailments (Randriamiharisoa et al., 2015; Razafindraibe et al., 2013). The dependence on remedies derived from medicinal plants is particularly important in these countries, as Madagascar, where modern medicine is often absent or simply too expensive (Novy, 1997): economic devaluation of the developing countries leads to higher prices of pharmaceuticals and makes medicinal plants and traditional medicine more attractive (Randriamiharisoa et al., 2015). Additionally, some prefer traditional medicine for several reasons including familiarity, tradition and perceived safety (van Andel and Carvalheiro, 2013).

Traditional Malagasy medicine makes use of a wide variety of plants to treat gastrointestinal disorders as diarrhea and intestinal parasites, which are particularly prevalent in rural areas of the country (Leutscher and Bagley, 2003). These diseases are rarely associated with mortality (gastrointestinal bleeding), but they cause significant morbidity as impaired physical and mental development (Areeshi et al., 2013).

Madagascar, located approximately 400 km off of the coast of Mozambique in southeastern Africa, is the fourth largest island in the world (Rasoanaivo, 1990). Due to such long isolation and to its tropical location, Madagascar is characterized by one of the highest rates of endemism and biodiversity in the world (Norscia and Borgognini-Tarli, 2006): indeed, current floristic calculations indicate that Madagascar hosts many endemic plant species (90% of vascular plant species and 96% of tree species) (Rasoanaivo, 1990; Razafindraibe et al., 2013). For this reason, it is not surprising that Malagasy flora can provide a wide variety of medicinal plants as an affordable alternative to expensive western medicine. In any case, only an estimated 10% of the Malagasy plant species has been screened for any biological activity until now (Hudson et al., 2000). The literature provides evidences on antiplasmodial and antimicrobial properties of different ethnomedicinal plant species from Madagascar (Norscia and Borgognini-Tarli, 2006; Rasoanaivo et al., 2004; Rakotoniriana et al., 2010), but the available survey on Malagasy medicinal plants is far from being exhaustive (Novy, 1997; Rasoanaivo, 1990).

Rural communities of Madagascar still practice and often prefer traditional medicine, especially for treating common and infectious diseases (Razafindraibe et al., 2013): in particular, about one third of the plants is used for the treatment of gastrointestinal disorders, one third in case of malaria/fever, and the remaining third in order to treat rheumatisms, cold, skin illnesses and inflammations (Norscia and Borgognini-Tarli, 2006). Moreover, reproductive, prenatal and postpartum health are the most frequently cited uses for medicinal plants in women's health.

Brachylaena, a member of the subfamily *Inulae*, is a genus of 20 species that occurs in tropical Africa and is questionably native of the Mascarenes. Five species occur in Madagascar, and *B. ramiflora* is widespread in highly disturbed areas, while in rainforest environments it is common from 500 to 2000 m of elevation, and it may

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persist in secondary vegetation, due to the corky bark and stool shoots. *B. ramiflora* (DC.) Humbert (Family *Asteraceae*) is also one of the most diffused medicinal plant of Madagascar, mainly used by local population as purgative and against stomach-ache; barks, however, are also used against blenorrhagia. In some biological tests the extracts were not distinctly toxic (RASOANAIVO et al., 1999). Depending on the ethnic group and language, several names for *B. ramiflora* occur: Hazotokana ("isolated tree") in Merina and Betsileo: 19 specimens; Mananotra/Mananitra in Betsileo, 7 specimens; Merana in Betsileo, 3 specimens; Kanda (near Ifanadiana), 2 specimens. As all of the Malagasy species of *Brachylaena*, the species *ramiflora* may reach 20 m in height and yields are very durable timber known to be termite resistant (trunks for construction) (CHATURVEDULA et al., 2002). Leaves drop when new leaves develop, either at or just after anthesis. The stalks to the capitula range considerably in length. The variant species with subglabrous leaves occurs scattered over the whole distribution area, with no obvious association with any particular habitat. The biological value of the genus *Brachylaena* has been documented (CHATURVEDULA et al., 2002): it has been described as a rich source of a high number of polyphenolic, organic and terpenic compounds with antioxidant, anti-inflammatory and antibacterial activity (VIEIRA et al., 1991).

Leaves are often the most used items for medicinal treatment, followed by bark and the entire plant, while decoctions and infusions are the most used methods of preparation (to be taken 3 times/day). Infusion is the extraction process of phytochemical compounds from plant material in a solvent as water, oil or alcohol, by allowing the material to remain suspended in the solvent over time; the process of infusion is different from decoction, which involves boiling the plant material (CAPASSO et al., 2006).

As the research on Malagasy medicinal plants resulted in the discovery of valuable drugs, the aim of this study was a preliminary phytochemical investigation on *B. ramiflora* leaves' and barks' infusions and decoctions, in order to characterize this species as source of biologically active compounds and adding new information on Malagasy ethnobotanical species, indentifying and quantifying the main biologically active compounds and their relative antioxidant activity. A better understanding of ethnopharmacological knowledge is crucial to Madagascar progress towards an improved self-sufficiency in health care and to the discovery of new natural treatments for globally significant diseases (RANDRIAMIHARISOA et al., 2015).

Materials and methods

Study area and plant material

The Maromizaha forest (18°56'49" S; 48°27'55" E) is located in Eastern Madagascar, in the Alaotra-Mangoro region (Moramanga district), within both the rural municipalities of Andasibe and Ambatovola. Maromizaha is a 1,880 ha New Protected Area of largely contiguous forest located 140 km east of Antananarivo and 225 km from Toamasina: it is bordered to the north by the Route Nationale 2, to the east by the Befody hills, to the west by the Madiorano River and to the south by the Ankazomirahavavy River (Fig. 1). The forest ranges between elevations of 794 m and 1,224 m. The Maromizaha forest is surrounded by three forest blocks, including the Special Reserve Analamazaotra to the northwest, Vohimana forest to the northeast and Vohidrazana forest to the east. This region of Madagascar is characterized by a tropical/sub-tropical climate tempered by altitude with high rainfall and a specific rainy season.

The analyzed samples of *B. ramiflora* (leaves and bark) are shown in Fig. 2. Leaves and bark of *B. ramiflora* were identified and authenticated by botanists of the Botanical and Zoological Park of Tsimbazaza (BZPT, Flora department) in Madagascar; a voucher specimen (reference: J.S. Miller, number 3760) was prepared and deposited in

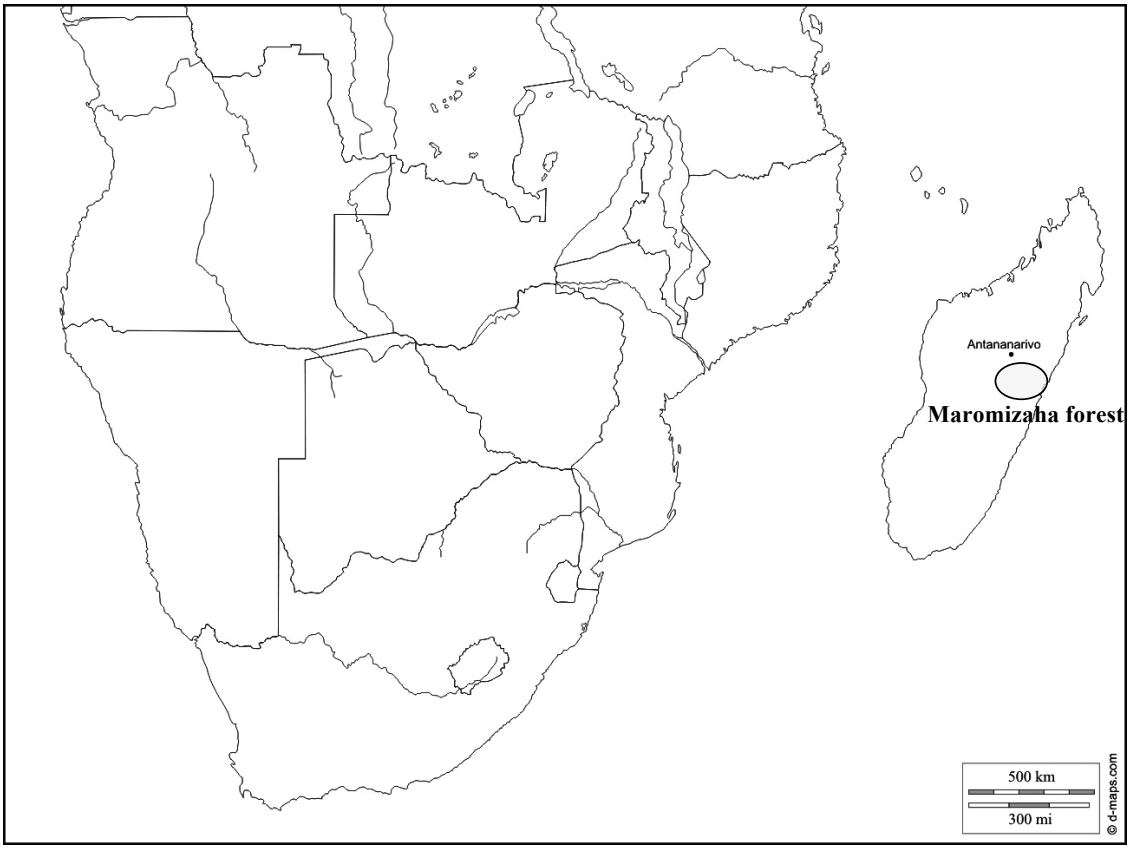

Fig. 1: Geographical location of *Brachylaena ramiflora*.

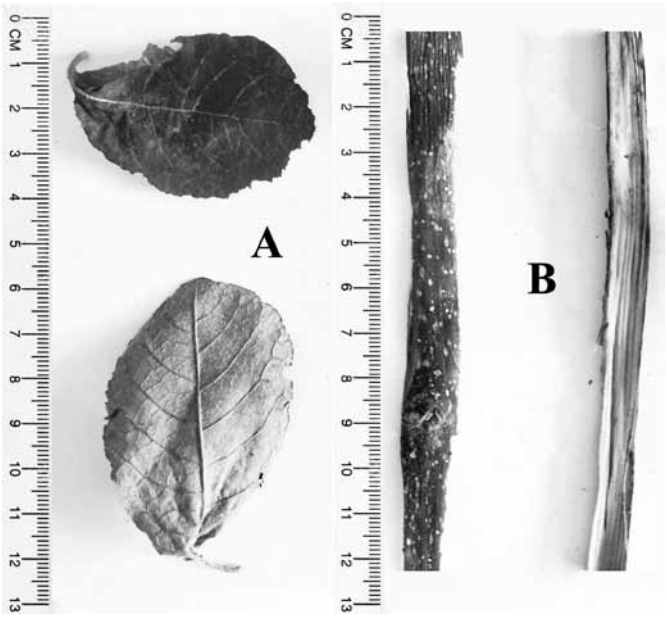

Fig. 2: Morphological traits of *Brachylaena ramiflora* leaves (A) and bark (B).

the herbarium section of the BZPT. Samples were collected in November 2015 in the region of Maromizaha forest where the plant is usually harvested for medicinal use by local population. Sampling was made in triplicate. According to local Malagasy traditions, infusions from leaves and decoctions from bark were prepared. In the infusion process, 200 mL of water was brought to an appropriate temperature (80 °C) and then poured over 5 g of leaves which were then allowed to steep in the liquid for 10 min. Each herbal infusion was filtered (Whatman Filter Paper, Hardened Ashless Circles, 185 mm Ø) and then stored at N.A., 4 °C and 95% R.H until analysis. In the decoction process, 20 g of bark were put in 200 mL of boiling water for 20 min; each sample was filtered (Whatman Filter Paper, Hardened Ashless Circles, 185 mm Ø) and then stored at N.A., 4 °C and 95% R.H until analysis.

Materials, solvents and chemicals

Sodium carbonate, Folin–Ciocalteu phenol reagent, sodium acetate, citric acid, potassium chloride, hydrochloric acid, iron(III) chloride hexahydrate, 2,4,6-tripyridyl-S-triazine, 1,2-phenylenediamine dihydrochloride (OPDA), all polyphenolic and terpenic standards, potassium dihydrogen phosphate, phosphoric acid and HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, ethanol, standards of organic acids and HPLC-grade formic acid were purchased from Fluka BioChemika, Buchs, Switzerland.

Ethylenediaminetetraacetic acid disodium salt was purchased from AMRESCO (Solon, OH, USA). Sodium fluoride was purchased from Riedel-de Haen (Seelze, Germany). Cetyltrimethylammonium bromide (cetrimide), ascorbic acid (AA) and dehydroascorbic acid (DHAA) were purchased from Extrasynthése (Genay, France). Milli-Q ultrapure water was produced by Sartorius Stedim Biotech mod. Arium (Sartorius, Göttingen, Germany).

Spectrophotometric analysis

The amount of total polyphenolic content (TPC) was determined following the Folin-Ciocalteu colorimetric method (Slinkard and Singleton, 1977) and results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW).

The total anthocyanin content (TAC) in the extracts was determined using the pH-differential method (Lee et al., 2005). TAC was expressed as milligrams of cyanidin-3-O-glucoside (C3G) per 100 grams of fresh weight ($mg_{C3G}/100\ g_{FW}$).

Antioxidant activity was evaluated by ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1999) and results were expressed as millimoles of ferrous iron (Fe^{2+}) equivalents per kilogram (solid food) of FW.

Chromatographic analysis

Sample preparation protocols for HPLC analysis

Small portions (2 mL) of obtained infusions and decoctions were filtered with circular pre-injection filters (0.45 µm, polytetrafluoroethylene membrane, PTFE) before HPLC-DAD analysis.

In the case of vitamin C analysis, a C_{18} cartridge for solid phase extraction (Sep-Pak® C-18, Waters, Milford, MA, USA) was used to absorb the polyphenolic fraction. Then, 250 µL of OPDA solution (18.8 $mmol{\cdot}L^{-1}$) were added to 750 µL of samples for DHAA derivatization into the fluorophore 3-(1,2-dihydroxyethyl)furo(3,4-b) quinoxalina-1-one (DFQ) (Gonzalez-Molina et al., 2008).

Standard calibration

The external standard method was used for quantitative determinations. Twenty mL aliquot manual injections were performed in triplicate for each concentration level. The calibration curves were obtained by plotting the peak area (y) of the compound at each level versus the sample concentration (x).

Apparatus and chromatographic conditions

An Agilent 1200 High Performance Liquid Chromatograph coupled to an Agilent UV-Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA), was used for the chromatographic analysis.

Five chromatographic methods were used: a KINETEX – C18 column (4.6 × 150 mm, 5 µm, Phenomenex, Torrance, CA, USA) was used to achieve the bioactive compound separation. Several mobile phases were used for the biomarker identification and UV spectra were recorded at different wavelengths. The chromatographic conditions of each method were reported in Tab. 1, while the main analytical method validation data are summarized in Tab. 2.

Identification and quantification of bioactive compounds in the extracts

All the samples were analyzed in triplicate, and standard deviations are given in order to assess the repeatability of the used methods. All single compounds were identified in samples by comparison and combination of their retention times and UV spectra with those of authentic standards in the same chromatographic conditions.

Total bioactive compound content (TBCC) was determined as the sum of the most important classes of selected biomarkers with an important role in the positive effects on human organism ("multimarker approach") (Mok and Chau, 2006). By single bioactive compound profile, phytochemicals were grouped into different bioactive classes to evaluate the contribution of each class to total phytocomplex composition. Biomarkers were selected for their demonstrated positive healthy properties and antioxidant activity by literature in relation to the use of this medicinal plant by local population. Five polyphenolic classes were considered: benzoic acids, catechins, cinnamic acids, flavonols, and tannins. Monoterpenes, organic acids, and vitamin C (as sum of ascorbic and dehydroascorbic acids) were also considered to obtain a complete analytical fingerprint. Mass spectrometry data of some selected phytochemicals in *B. ramiflora*

Tab. 1: Chromatographic conditions of each used method (DONNO et al., 2015c).

Method	Compounds of interest	Stationary phase	Mobile phase	Flow *(mL min^{-1})*	Wavelenght *(nm)*
A	cinnamic acids, flavonols	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: 10 mM KH_2PO_4/H_3PO_4, pH=2.8 B: CH_3CN	1.5	330
B	benzoic acids, catechins, tannins	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1 v/v/v), pH=2.5 B: $CH_3OH/HCOOH$ (100:0.1 v/v)	0.6	280
C	monoterpenes	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: H_2O B: CH_3CN	1.0	210, 220, 235, 250
D	organic acids	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: 10 mM KH_2PO_4/H_3PO_4, pH=2.8 B: CH_3CN	0.6	214
E	vitamins	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: 5 mM $C_{16}H_{33}N(CH_3)_3Br$/50 mM KH_2PO_4, pH=2.5 B: CH_3OH	0.9	261, 348

Elution conditions
Method A / gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min (2 min conditioning time)
Method B / gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min (2 min conditioning time)
Method C / gradient analysis: 30% B to 56% B in 15 min + 56% B in 2 min (3 min conditioning)
Method D / isocratic analysis ratio of phase A and B: 95:5 in 13 min (2 min conditioning time)
Method E / isocratic analysis: ratio of phase A and B: 95:5 in 10 min (5 min conditioning time)

decoctions and infusions were reported in supplementary materials (Fig. S1). All the results were expressed as mg per 100 g of fresh weight (FW).

Statistical Analysis
All samples were prepared and analyzed in triplicate. Results were subjected to t-Student test and ANOVA test for mean comparison (SPSS 22.0 Software) and HSD Tukey multiple range test ($P < 0.05$). The relationships between the TPC and antioxidant activity were investigated using Pearson's correlation coefficient (R).

Results and discussion

The beneficial effects of bark and leaves of Malagasy medicinal plants on human health have already been established in several studies (RANDRIAMIHARISOA et al., 2015): in particular, the amount of total phenolics could be responsible for a great number of these effects. Nevertheless, studies on tree-species plant bioactive compounds (botanicals) are still very scarce: in this study, the phytochemical value of infusions and decoctions of different parts of *Brachylaena ramiflora* was investigated by chromatographic and spectrophotometric analyses (by determination of vitamins and organic acids, simple phenolics, flavonoids, anthocyanins and tannins, and of the antioxidant activity). This is one of the first reports which evaluates the *B. ramiflora* leaf infusion and bark decoction for their chemical parameters, phytochemical profile and antioxidant activity.

Total phenolics and anthocyanins
Phenols and related compounds, as anthocyanins, have antioxidant potential owing to hydroxyl groups, which allow free ion pairs to be donated easily. The TPC determination in plant infusions and decoctions was carried out by the Folin-Ciocalteu method, which depends on electron transfer from phenolic compounds to the Folin-Ciocalteu reagent in alkaline medium (ERENLER et al., 2016; DONNO et al., 2015b), while the TAC was directly determined using the pH-differential method: the colored oxonium form of anthocyanin predominates at pH 1.0, and the colorless hemiketal form at pH4.5; the pH-differential method is based on the reaction producing oxonium forms (DONNO et al., 2015d).

TPC and TAC, spectrophotometrically estimated in the decoctions and infusions, are shown in Tab. 3. The results of the TPC revealed that infusions and decoctions have different phenolic content which ranged significantly from 11.15±3.17 to 96.10±1.77 $mg_{GAE}/100\ g_{FW}$, respectively. With regard to the TAC, the infusion has shown a significantly higher content (27.86±13.99 $mg_{C3G}/100\ g_{FW}$) compared to decoction (3.03±0.64 $mg_{C3G}/100\ g_{FW}$). The results demonstrated that the highest polyphenol contents were found in the decoctions as reported in other studies (RAZAKARIVELO et al., 2015; KARIMI et al., 2010), even if these polyphenolic compounds were not anthocyanins: the preparation method could have influence on polyphenolic components, which leads to more efficient extraction for decoction method, but could also affect the phenolic molecules during the continuous heat to which decoctions were subjected as reported by AMMAR et al. (2015).

Phytochemical profile and phytocomplex
Synergistic or additive therapeutic effects of several phytochemicals, rather than a single compound, could contribute to disease prevention and reduce the risk of addiction and toxicity, producing a more complete and less drastic pharmacological effect than that of one or a few of its components taken separately: the so-called phytocomplex consists of a combination of different substances, both active principles and other plant components, which contribute to the overall therapeutic effect (DONNO et al., 2015a). Since the biological activity of *B. ramiflora* infusions and decoctions is due to the sum of their bioactive components (phytocomplex), chemical composition of these preparations was analyzed by HPLC fingerprint: the main constituents identified in the present study (polyphenolic and terpenic compounds, organic acids, and vitamins) are known bioactive compounds. Sixteen and twenty-three biomarkers were identified in the *B. ramiflora* leaf infusions and bark decoctions, respectively (Tab. 4): the main compounds identified by HPLC-DAD in the infusions were quinic acid (334.55±0.99 mg/100 g_{FW}), chlorogenic acid (208.27± 7.74 mg/100 g_{FW}), and γ-terpinene (144.19±1.00 mg/100 g_{FW}), while the major components in the decoctions were castalagin (2002.64± 13.96 mg/100 g_{FW}), citric acid (1171.81±1.05 mg/100 g_{FW}), and chlorogenic acid (646.44±2.31 mg/100 g_{FW}).
Overall, infusions and decoctions have shown different phenolic profile. Seven (leaf infusions) and fourteen (bark decoctions) phe-

Tab. 2: Main validation parameters of the used methods for each calibration standard (DONNO et al., 2015c).

Method	Class	Standard	ID code[a]	Retention time (t_R) (min)	Wavelenght (nm)	Calibration curve equation	R^2	Calibration curve range (mg L^{-1})	LOD[b] (mg L^{-1})	LOQ[c] (mg L^{-1})
A	Cinnamic acids	caffeic acid	1	4.54	330	y = 59.046x + 200.6	0.996	111 - 500	0.305	1.016
		chlorogenic acid	2	3.89	330	y = 13.583x + 760.05	0.984	111 - 500	0.940	3.134
		coumaric acid	3	6.74	330	y = 8.9342x + 217.4	0.997	111 - 500	2.907	9.690
		ferulic acid	4	7.99	330	y = 3.3963x - 4.9524	1.000	111 - 500	1.245	4.150
	Flavonols	hyperoside	5	10.89	330	y = 7.1322x - 4.583	0.999	111 - 500	3.372	11.241
		isoquercitrin	6	11.24	330	y = 8.3078x + 26.621	0.999	111 - 500	0.252	0.840
		quercetin	7	17.67	330	y = 3.4095x - 98.307	0.998	111 - 500	4.055	13.518
		quercitrin	8	13.28	330	y = 2.7413x + 5.6367	0.998	111 - 500	5.456	18.187
		rutin	9	12.95	330	y = 6.5808x + 30.831	0.999	111 - 500	2.937	9.790
B	Benzoic acids	ellagic acid	10	18.65	280	y = 29.954x + 184.52	0.998	62.5 - 250	0.611	2.035
		gallic acid	11	4.26	280	y = 44.996x + 261.86	0.999	62.5 - 250	0.435	1.451
	Catechins	catechin	12	10.31	280	y = 8.9197x + 66.952	1.000	62.5 - 250	2.343	7.809
		epicatechin	13	14.30	280	y = 12.88x - 43.816	0.999	62.5 - 250	0.763	2.543
	Tannins	castalagin	14	16.35	280	y = 4.236x - 8.535	1.000	62.5 - 250	1.009	3.363
		vescalagin	15	17.25	280	y = 4.939x - 1.232	1.000	62.5 - 250	0.603	2.010
C	Monoterpenes	limonene	16	3.35	250	y = 0.1894x - 5.420	0.999	125 - 1000	8.654	28.847
		phellandrene	17	3.57	210	y = 8.783x - 145.3	0.998	125 - 1000	0.562	1.874
		sabinene	18	3.45	220	y = 18.14x - 1004	0.998	125 - 1000	0.094	0.314
		γ-terpinene	19	3.28	235	y = 0.4886x - 23.02	0.999	125 - 1000	17.577	58.590
		terpinolene	20	4.83	220	y = 26.52x + 876.8	0.999	125 - 1000	0.241	0.804
D	Organic acids	citric acid	21	5.30	214	y = 1.0603x - 22.092	1.000	167 - 1000	18.805	62.682
		malic acid	22	4.03	214	y = 1.415x - 80.254	0.996	167 - 1000	15.721	52.404
		oxalic acid	23	7.85	214	y = 6.4502x + 6.1503	0.998	167 - 1000	0.550	1.835
		quinic acid	24	3.21	214	y = 0.8087x - 38.021	0.998	167 - 1000	26.106	87.021
		succinic acid	25	3.46	214	y = 0.9236x - 8.0823	0.995	167 - 1000	7.135	23.783
		tartaric acid	26	5.69	214	y = 1.8427x + 15.796	1.000	167 - 1000	8.520	28.401
E	Vitamins	ascorbic acid	27	4.14	261	y = 42.71x + 27.969	0.999	100 - 1000	0.836	2.786
		dehydroascorbic acid	28	3.41	348	y = 4.1628x + 140.01	0.999	30 - 300	1.095	3.649

[a]ID code = identification code; [b]LOD = limit of detection; [c]LOQ = limit of quantification

Tab. 3: Total polyphenolic content (TPC), antioxidant activity, and total anthocyanin content (TAC) data in the analyzed plant extracts.

	TPC *Mean value±SD* ($mg_{GAE}/100\ g_{FW}$)	**Antioxidant activity** *Mean value±SD* (mmol Fe^{2+}/kg)	**TAC** *Mean value±SD* ($mg_{C3G}/100g_{FW}$)
Infusion	11.15±3.17[a]	1.07±0.03[a]	27.86±13.99[b]
Decoction	96.10±1.77[b]	6.42±0.08[b]	3.03±0.64[a]

Mean value and standard deviation of each sample is given (N = 3). Different letters in superscript for each sample indicate the significant differences at $P < 0.05$.
[a]GAE = gallic acid equivalents; [b]C3G = cyanidin-3-O-glucoside; [c]FW = fresh weight

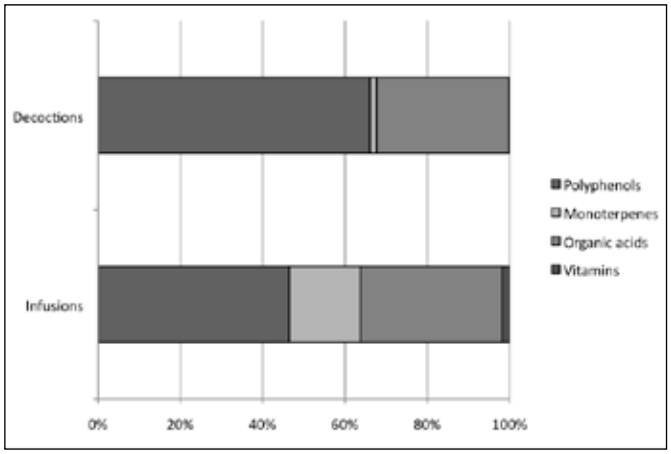

Fig. 3: Phytocomplex representation of the *Brachylaena ramiflora* extracts. Mean value of each analyzed sample is given (N = 3).

nolic compounds were detected, identified, and quantified by their retention times and UV spectra compared with those of analytical standards analyzed in the same chromatographic conditions using a HPLC-DAD: polyphenols represented 46.43% of the infusion phytocomplex and 66.08% of the decoction phytocomplex (Fig. 3), accor-ding to other researches (Rasoanaivo et al., 2004; Razakarivelo et al., 2015). Phenolic compounds may be found in plant infusions and decoctions by boiling water particularly due to solution of hydrolysable tannins and flavonoids and water-soluble lignin fragments solved under acidic conditions (Erenler et al., 2016). The main polyphenolic compounds found in infusions and decoctions of *B. ramiflora* leaves and bark were phenolic acids (31.88%) and tannins (28.95%), respectively (Tab. 5). Concerning the phenolic acids, they were identified as cinnamic and benzoic acids and tentatively identified as caffeic, chlorogenic, coumaric, and ferulic acids and ellagic and gallic acids, respectively. The presence of phenolic acids, as caffeic, ferulic and coumaric acids, has been already reported in plant material of different species with the same therapeutic effects (Ammar et al., 2015). The present study did not report the presence of phenolic acid derivatives even if these compounds are present mainly as water soluble glycosides. Moreover, the leaf infusion has revealed a considerably lower tannin content than the bark decoction: indeed, bark was expected to contain higher mass fraction of tannins since these compounds have a defensive role (Majic et al., 2015). The presence of tannins in adequate amounts in bark extracts could be advantageous as they are able to very effectively quench free radicals (Ammar et al., 2015). The identified biomarkers have a pharmaceutical and medicinal importance: in general, the antioxidant effects of

Tab. 4: Phytochemical fingerprint of analyzed samples.

Class	**Bioactive marker**	**Infusion** *Mean value±SD* ($mg/100g_{FW}$)	**Decoction** *Mean value±SD* ($mg/100g_{FW}$)
Cinnamic acids	caffeic acid	13.10±0.63	5.44±0.34
	chlorogenic acid	208.27±7.74	646.44±2.31
	coumaric acid	90.20±8.30	426.86±2.52
	ferulic acid	n.d.	58.30±3.13
Flavonols	hyperoside	n.d.	12.62±2.39
	isoquercitrin	9.67±0.54	418.39±17.95
	quercetin	120.44±0.86	293.47±7.47
	quercitrin	n.d.	54.44±0.93
	rutin	n.d.	85.98±7.17
Benzoic acids	ellagic acid	13.13±0.41	447.95±4.82
	gallic acid	21.45±1.36	58.32±8.32
Catechins	catechin	n.d.	149.24±6.43
	epicatechin	n.d.	n.d.
Tannins	castalagin	n.d.	2002.64±13.96
	vescalagin	n.d.	71.77±1.21
Monoterpenes	limonene	n.d.	n.d.
	phellandrene	13.83±0.40	116.67±1.17
	sabinene	20.37±0.59	n.d.
	γ-terpinene	144.19±1.00	n.d.
	terpinolene	9.79±0.94	n.d.
Organic acids	citric acid	n.d.	1171.81±1.05
	malic acid	n.d.	476.41±1.57
	oxalic acid	4.46±0.30	85.18±0.65
	quinic acid	334.55±0.99	16.00±0.18
	succinic acid	34.83±0.15	14.15±0.10
	tartaric acid	n.d.	536.80±1.35
Vitamins	ascorbic acid	12.38±0.09	12.51±0.06
	dehydroascorbic acid	7.31±0.14	0.71±0.04

Mean value and standard deviation of each sample is given (N = 3).
[a]n.d. = not detected; [b]FW = fresh weight

phenolic compounds have been studied in relation to the prevention of coronary diseases and cancer, as well as age-related degenerative brain disorders (Canterino et al., 2012). In addition, phenolic compounds, associated with antioxidant activity, play an important role in stabilizing lipid peroxidation (Beyhan et al., 2010).
Terpenic compounds were dominant in *B. ramiflora* infusions in contrast to the decoctions: as shown in Tab. 5, monoterpenes represented 17.33% of the infusion phytocomplex and 1.63% of the decoction phytocomplex. The main terpenic compounds of infusions were γ-terpinene (144.19±1.00 mg/100 g_{FW}) and sabinene (20.37±0.59 mg/100 g_{FW}) followed by phellandrene (13.83±0.40 mg/100 g_{FW}), while the only identified monoterpene of decoctions was phellandrene (116.67±17.17 mg/100 g_{FW}) as reported in Tab. 4. This difference in terpenic compound composition is probably due to the different extraction methods (Piry et al., 1995), in particular to the temperature: these molecules are volatile compounds with anti-inflammatory activities (de Cassia da Silveir et al., 2013) and the obtained results could explain the differences in the antioxidant activity between infusions and decoctions. The monoterpene separation was very dif-

Tab. 5: Phytocomplex of *Brachylaena ramiflora* infusions and decoctions.

	Infusions		Decoctions	
Bioactive class	*Mean value±SD* ($mg/100g_{FW}$)	*phytocomplex percentage* (%)	*Mean value±SD* ($mg/100g_{FW}$)	*phytocomplex percentage* (%)
Cinnamic acids	$311.56±8.53^{e}$	28.69	$1137.04±6.34^{f}$	15.87
Flavonols	$130.11±0.58^{c}$	11.98	$864.90±14.86^{e}$	12.07
Benzoic acids	$34.57±1.62^{b}$	3.18	$506.28±3.81^{d}$	7.07
Catechins	n.d.	/	$149.24±6.43^{c}$	2.08
Tannins	n.d.	/	$2074.41±13.17^{g}$	28.95
Anthocyanins	$27.86±5.17^{ab}$	2.57	$3.03±0.64^{a}$	0.04
Monoterpenes	$188.17±0.33^{d}$	17.33	$116.67±1.17^{b}$	1.63
Organic acids	$373.85±0.90^{f}$	34.43	$2300.35±1.80^{h}$	32.10
Vitamins	$19.69±0.16^{a}$	1.81	$13.21±0.02^{a}$	0.18
TBCC	1085.81±12.21		7165.13±11.64	

Mean value and standard deviation of each sample is given (N = 3). Different letters in superscript for each sample indicate the significant differences at $P < 0.05$.
[a]n.d. = not detected; [b]FW = fresh weight

ficult (not so much high resolution), and for this reason the separation was also achieved by four different wavelengths.

Organic acids and vitamin C (as sum of ascorbic acid and dehydroascorbic acid) are another important antioxidant components with multi-purpose uses in pharmacology (EYDURAN et al., 2015). In *B. ramiflora* infusions they represented 34.43% and 1.81% of the total phytocomplex, respectively; in the decoctions, instead, organic acids represented 32.10% of the phytocomplex, while vitamin C participated in the phytocomplex with only 0.18% (Fig. 3) because of the high extraction temperature that degrades the most heat-sensitive molecules.

Antioxidant activity

Antioxidant capacity is widely used as a parameter for medicinally bioactive and functional components in plant material and derived-products: the reducing capacity of specific compounds may serve as a significant indicator of total potential antioxidant activity (DONNO et al., 2013). The mechanisms of antioxidant activity of polyphenolic compounds and vitamins, as flavonoids and vitamin C, are well discussed but the mechanisms and structural requirements have not been fully understood (AMIĆ et al., 2003). The antioxidant properties of monoterpenes and organic acids have been also referred to by several authors (EYDURAN et al., 2015).

In this study, *B. ramiflora* leaf infusions and bark decoctions have been evaluated for their activity as free radical scavengers by FRAP assay. The presence of antioxidant molecules in plant samples brings about the reduction of the Fe^{3+} complex to the ferrous form: in this assay, the yellow color of the test solution changes to different shades of green and blue depending on the reducing power of antioxidant samples. The antioxidant compounds, present in analyzed extracts, reduced the Fe^{3+} complex to the Fe^{2+} form in different way depending on extraction method: the antioxidant capacity ranged from 1.07±0.03 mmol Fe^{2+}/kg (infusions) to 6.42±0.08 mmol Fe^{2+}/kg (decoctions) (Tab. 3), according to similar studies (KARIMI et al., 2010).

Most of the identified compounds possess hydroxyl groups which have the ability of formation complexes with iron metal: the hydroxyl groups of the molecules donate electron pairs to the iron metal with a coordinate covalent bond to form metal complexes. The antioxidant activity of *B. ramiflora* leaves and bark could be attributed to the hydroxyl groups of the molecules that donate electron pairs to the metal, as also reported by ERENLER et al. (2016).

The high Pearson's correlation coefficients between the polyphenolic content and antioxidant activity ($R_{infusions} = 0.99$ and $R_{decoctions} = 0.62$) confirmed a strong positive linear relationship between these two variables according to similar researches (MAJIC et al., 2015).

Conclusions

Folk medicine represents an important tool to spot plants of pharmacological interest, since it can indicate potential sources of bioactive compounds. It can be inferred that the forest of Maromizaha is a source of important raw materials for plant-derived pharmaceuticals. Medicinal plant exploitation have a link with biodiversity conservation. The valorization of medicinal plants may increase local incentives to preserve and manage the habitat. It is hoped that further studies will generate an interest in the proper sustainable production, processing, and commercialization of *B. ramiflora* for medicinal purposes. Indeed, according to the results of this study, it may be concluded that *B. ramiflora* could be considered as a promising source of natural antioxidants that may contribute to health benefits: the analyzed preparations revealed an important polyphenol content, in particular flavonoids and tannins, and the decoctions presented the highest levels. The phytochemical profile was also characterized by the presence of monoterpenes, organic acids and vitamin C.

Furthermore, agreements between local institutions and pharmaceutical companies may encourage a further development of prospective medicines and natural remedies based on *B. ramiflora* and other local medicinal plants.

As the quantity of wild growing plant species is continuously declining, a major effort should be done to strengthen conservation policies and strategies. In this perspective, this preliminary survey would give a contribute to the knowledge of Malagasy flora: the outcomes of this preliminary phytochemical investigation may provide a contribution to the identification and quantification of lead compounds responsible for traditional therapeutic claims, but a further quantitative evaluation on the basis of their chemical structures with HPLC coupled to mass spectrometry is necessary.

Finally, further studies providing more ecophysiological and pharmacological information are necessary to have a more complete picture on *B. ramiflora*, highlighting the importance of biodiversity on the health and wellbeing of local communities.

Declaration of interest
The authors declare that they have no conflict of interest.

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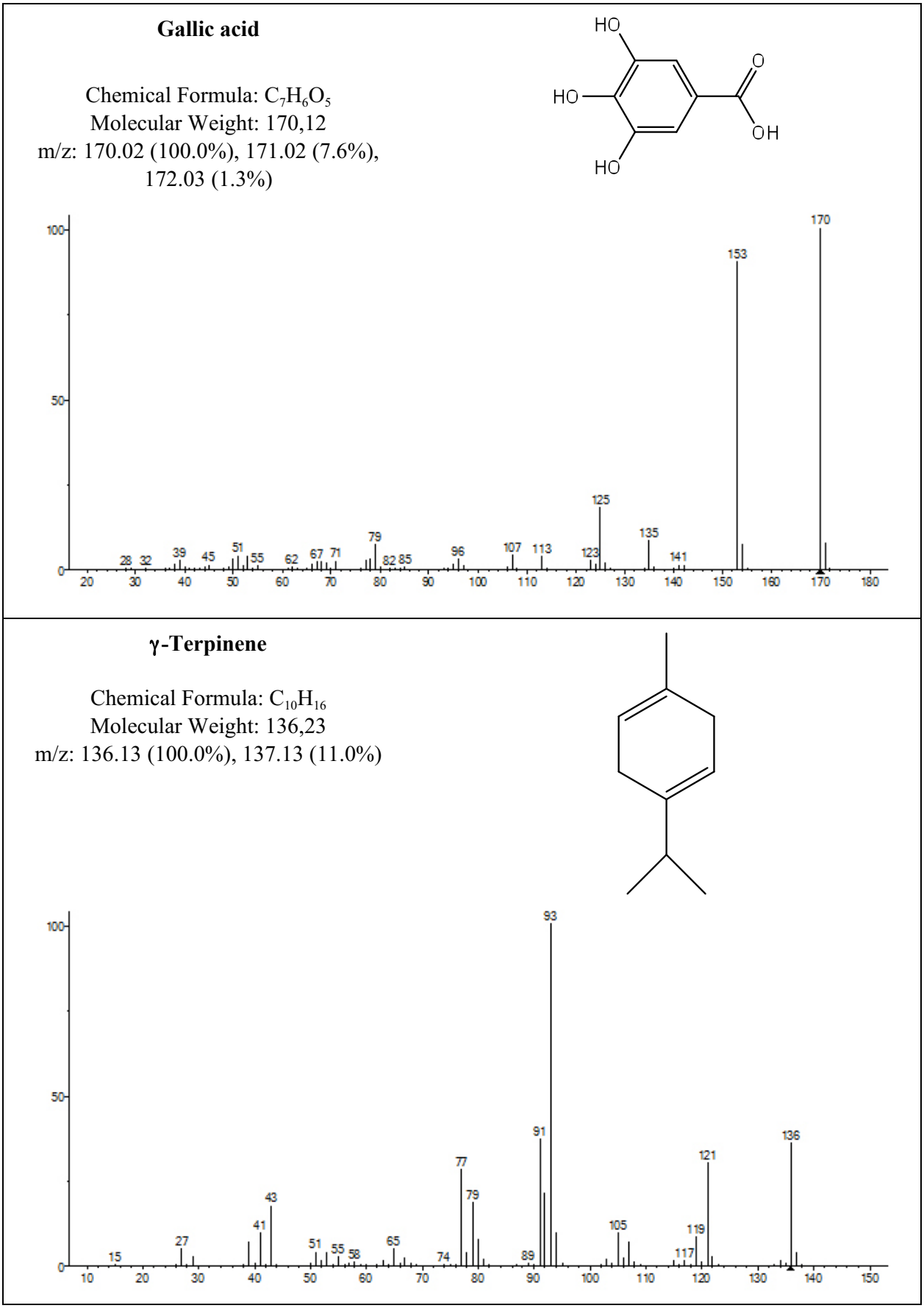
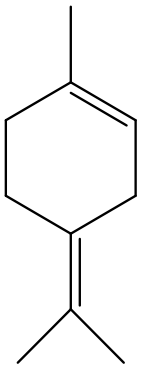
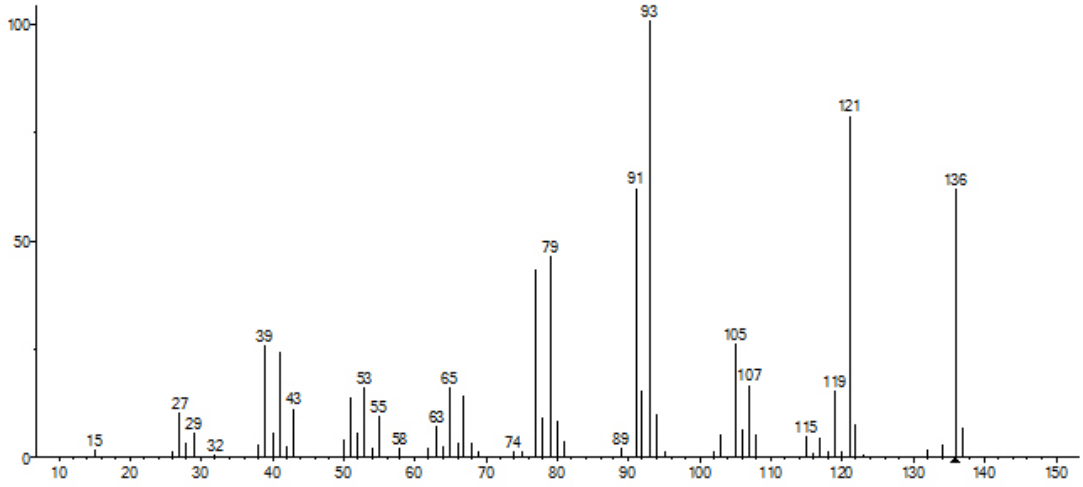
Fig. S1: Mass spectrometry data of some selected phytochemicals in *B. ramiflora* decoctions and infusions



Terpinolene

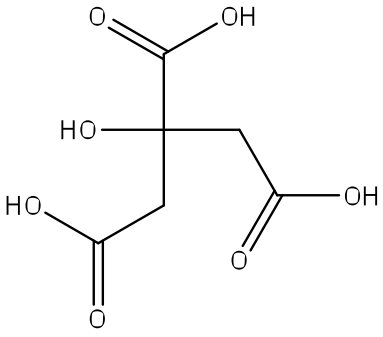
Chemical Formula: $C_{10}H_{16}$
Molecular Weight: 136,23
m/z: 136.13 (100.0%), 137.13 (11.0%)

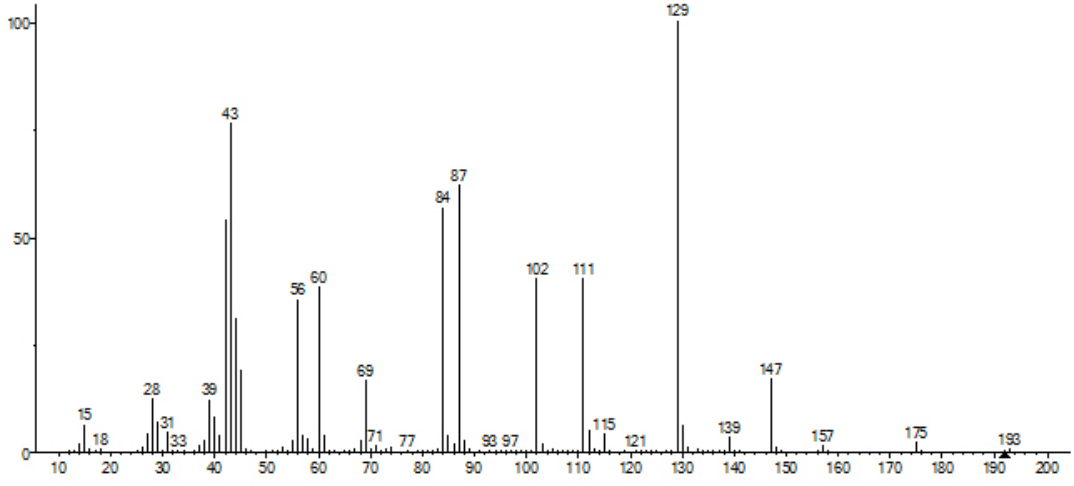




Citric acid

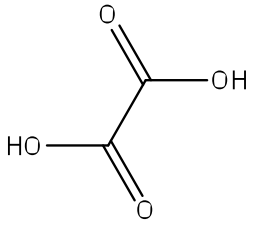
Chemical Formula: $C_6H_8O_7$
Molecular Weight: 192,12
m/z: 192.03 (100.0%), 193.03 (6.8%), 194.03 (1.6%)

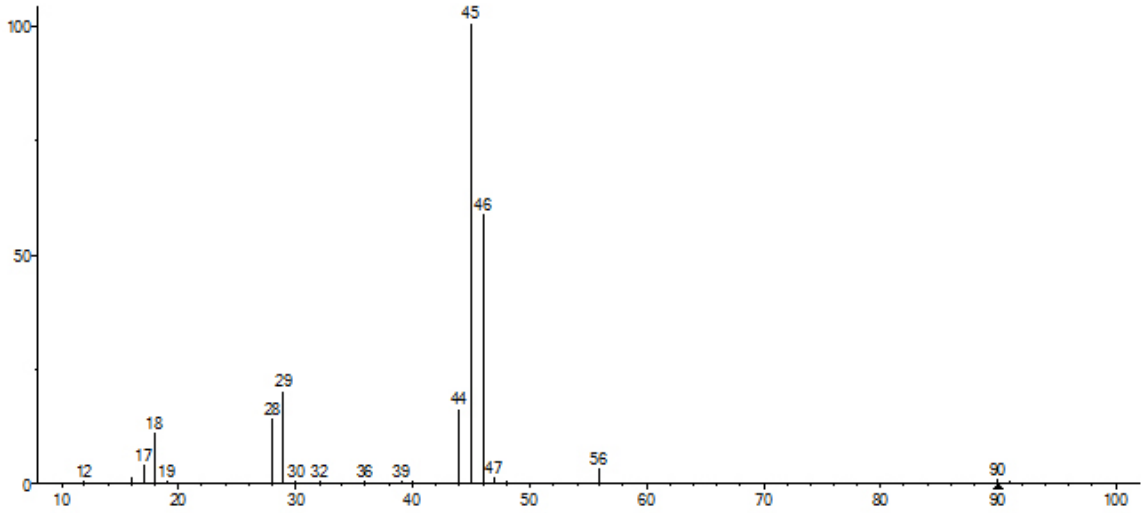




Oxalic acid

Chemical Formula: $C_2H_2O_4$
Molecular Weight: 90,03
m/z: 90.00 (100.0%), 91.00 (2.3%)





Tartaric acid

Chemical Formula: $C_4H_6O_6$
Molecular Weight: 150,09
m/z: 150.02 (100.0%), 151.02 (4.6%), 152.02 (1.3%)