Scientific Research Publishing

American Journal of Plant Sciences, 2017, 8, 1560-1571
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Chemical Composition, Antioxidant and Antimicrobial Activity of the Oil and Plant Extract *Myrocarpus frondosus* Allemão

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How to cite this paper: de Santi, I.I., Gatto, D.A., Machado, M.R.G., dos Santos, P.S.B. and Freitag, R.A. (2017) Chemical Composition, Antioxidant and Antimicrobial Activity of the Oil and Plant Extract *Myrocarpus frondosus* Allemão. *American Journal of Plant Sciences*, **8**, 1560-1571.
https://doi.org/10.4236/ajps.2017.87108

Received: April 20, 2017
Accepted: June 13, 2017
Published: June 16, 2017

Abstract

The objective of this study was to evaluate the chemical profile of extracts (aqueous and ethanol) and the essential oil of *Myrocarpus frondosus* Allemão, the sensitivity of strains of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Listéria monocytogenes*, front the extracts and essential oil, by means of the microdilution method in broth, the antioxidant activity by the ABTS method, and the content of phenolic compounds present in the extracts and oil. For the preparation of extracts from plant leaves used with ethanol and water, then separated, the chemical identification of compounds was performed by high-performance liquid chromatography (CLAE-DAD) and gas chromatography coupled to mass spectrum (CG/MS). With the chemical analysis of the extracts obtained the presence of the major compound rutin, and oil major compound was found germacrene B. In the microdilution method in broth, oil and extracts showed inhibition against all the bacteria tested in the concentrations 1 mg/ml to 0.25 mg/ml, except for *Staphylococcus aureus* at a concentration of 0.25 mg/ml of the essential oil and trans-caryophyllene. The results of Minimum Bactericidal Concentration showed that the essential oil had bactericidal activity at a concentration of 1mg/ml for all bacteria tested and trans-caryophyllene at the same concentration only for *Listeria monocytogenes*. In relation to the essential oil, antioxidant activity showed higher radical reduction capacity of 40.92% and the content of phenolic compounds ethanol extract showed more 12.72%. The *in vitro* results support the conclusion that the essential oil is very promising both in antimicrobial action as antioxidant activity and the leaf extracts on antioxidant activity.

Keywords

Myrocarpus frondosus Allemão, Antimicrobial, Antioxidant

1. Introduction

Medicinal plants have the largest pharmaceutical source that exists and the use of them is as old as human life. Before the nineteenth century, the plants were the main resource used in traditional medicine [1]. The use of herbal substances stands out both as a therapeutic agent as raw material for synthesis of drugs. This practice was valued by the national pharmaceutical market international and research and development of new drugs [2]-[7].

So today many scientists from different areas are using substances extracted from medicinal plants due to its high efficacy against pathogens and its use was already proven use in traditional medicine [1].

However, the widespread use of drugs, especially non-prescription, has led to a loss of effectiveness of action against pathogenic microorganisms [7]. Therefore, Fabri [8] describes that plants that have antibacterial activity are extremely important, given that many bacteria are resistant to the latest antibiotics.

According to Duarte [9] in addition to antimicrobial activity, have also been sought new sources of antioxidants derived from natural products. Moreover, with increasing restriction of the use synthetic antioxidants due to its toxicity reported recently that might involve many risks to health, including cancer [10], thus the scientific study has focused for the identification of novel antioxidant compounds from different sources, such as trees, herbs and spices, among others [11] [12].

According to Calixto [13] Brazil, it has highlighted to present the largest storehouse of biodiversity on the planet, with great potential for research and development of new products from medicinal plants. So if found in nature, plants, we are aiming to discover new compounds with antimicrobial and antioxidant activity.

Traditional medicine has using parts of *Myrocarpus frondosus* Allemão plant, to treat wounds and bruises, as an expectorant, and other lesions of the respiratory system, anti-inflammatory among other uses [14] [15].

In this context, this study aims to evaluate the antimicrobial activity, antioxidant of aqueous and ethanolic extracts of leaves, and essential oil from plant seeds *Myrocarpus frondosus* Allemão (Cabreúva) and the extremely important for pharmaceutical and food industry and identify main chemical constituents present in aqueous and ethanolic extracts of the leaves and the essential oil of the seeds.

2. Materials and Methods

2.1. Plant Material

The leaves and seeds were collected on the upper slopes of the Northeast in the

state of the Rio Grande do Sul. In the anatomy laboratory of wood, the material was dried at 35˚C in an air circulation greenhouse and stored in climate–controlled chamber. For identify the species, plant samples were sent to the Herbarium of Forest Engineering at the Federal University of Santa Maria–UFSM, and identified and cataloged under number HDCF n˚ 6215.

2.2. Preparation of Extracts: Aqueous and Ethanolic

For obtaining the aqueous raw extract were used 25 g of crushed dry leaves in knife mills and 100 mL of distilled water. The plant sample and the solvent were placed in a container with 500 mL and kept under stirring of 600 RPM, heating plate with an oil bath at a temperature of 60˚C for 24 hours. The process was repeated until obtaining a clear extract. In the end of extraction, the extract was frozen at −80˚C for subsequent lyophilization, thereby obtaining the dry extract. For obtaining the ethanolic extract followed by the same extraction procedure as above, the solvent used was ethanol. Already the ethanolic extract it evaporated in rotary evaporator to obtain the dry extract. The dried samples were placed in small vials hermetically sealed for later use in chromatographic analysis and *in vitro* tests.

2.3. Essential Oil Extraction

The essential oil was extracted by hydrodistillation method. Was weight 100 g of the previously crushed seed in a blender along with 1500 mL distilled water and ad ded to a flask attached to Clevenger apparatus. The extraction was performed as described in the Brazilian Pharmacopoeia [16].

2.4. Chemical Analysis

For the identification of metabolites present in the essential oil the same were subjected to chromatographic analysis in equipment GC/MS, brand Shimadzu QP2010, equipped with a splitter split/splitless. With an Rtx-5MS Restek (30 m × 0.25 mm × 0.25 microns) capillary column under the following chromatographic conditions: Helium gas carrier obtained by electron impact fragments to a power of 70 eV rate of 1.2 ml/min, 1:50 split flow and the volume of injected sample of 1 ul. Programmed oven temperature: initial temperature was 40˚C with a heating ramp of 10˚C/min to 280˚C and remained stable at this temperature for 10 minutes. Subsequently the temperature was increased at a rate of 10˚C/minute to 300˚C for a total time of 41 minutes with a injector temperature 250˚C, the interface temperature 300˚C, the compounds were analyzed; NIST08 using GC/MS library.

The ethanolic and aqueous extracts were analyzed by HPLC-DAD (Shimadzu). The reverse phase chromatography was conducted with Phenomenex C-18 column (4.6 mm × 250 mm), using as mobile phase A (2% acetic acid) and B (methanol) at a flow rate 0.8 ml/min and injection volume of 40 µL according to the elution method and Piglet gradient Evaristo and Leitão [17] slightly modified. The extracts were conducted in triplicate, for the presence of rutin and

confirmation of the presence of this substance was given by comparison of retention times and absorption spectra (in the range of 230 to 400 nm) peaks. Rutin standard curve was prepared ranging from 5 to 40 μg/mL for quantification.

2.5. Microorganisms

The antimicrobial activity of the extracts, oil essential, rutin and caryophyllene oxide were individually tested with four patterns strains: *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Listeria monocytogenes* ATCC 7644 and *Salmonellatyhimurium* ATCC 13311.

2.6. Chemical Standards

The standards used were the Trans-Caryophyllene, ≥98.5% 001.321.406 lot and Rutin Hydrated, ≥94.0%, BCBH0339V lot, purchased from Sigma-Aldrich.

2.7. Preparation of the Inoculum

In a test tube, containing saline 0.85% to prepare suspensions of the test strains that were standardized according to Mac Farland 0.5 range, corresponding roughly to the concentration of 1.5×10^8 Colony Forming Units (CFU/mL). For standardize the optical density of the inoculum was read in a spectrophotometer at a wavelength $\lambda = 625$ nm in an absorbance of 0.08 - 0.10 according to NCCLS (2003) with modifications [18].

2.8. Determination of Minimum Inhibitory Concentration (MIC)

The broth susceptibility test was performed using the method NCCLS (2003) with modifications, for the determination of MIC. All tests were conducted in trypticase soy broth (TSB). To perform the antimicrobial activity, two standards were used, rutin, major compound of the extracts and the trans-caryophyllene only major compound of essential oil available for purchase, extracts (aqueous e ethanolic) e oil essential. Samples and standards were diluted in DMSO with the initial concentration of 1mg/ml. Prepared a bacterial suspension with a concentration of 5×10^5 CFU/mL. In a total volume of 100 μl of the suspension of standardized microorganisms they were inoculated into each well, and then added to 100 μl of the sample solution. The plates were incubated at 37˚C for a period of 24 h. The MIC was calculated as the highest dilution, showing complete inhibition of standard strain used.

The inhibition of control were used antibiotics against Gram+ and Gram−, positive and negative control and in microdilution method was used dimethylsulfoxide (DMSO).

2.9. Determination of Minimal Bactericidal Concentration (MBC)

The results of the MIC, the wells showing complete absence of growth in each well were identified and 10 μl of each well were transferred to a Mueller Hinton Agar plates and incubated at 37˚C for 24 h. The complete absence of growth, was considered as the minimum bactericidal concentration.

2.10. Antioxidant Activity

For the determination of total phenols used the method described by Lachman [19]. Was defined, also the antioxidant capacity of the samples, and thus extract the oil capacity and reduce ABTS radical according to the spectrophotometric method [20].

The radical ABTS is obtained after the reaction ABTS (0.0192 g) of potassium persulfate (5 ml) incubated at room temperature (±25˚C) and in the dark for 24 h. Once the ABTS radicals formed diluted with ethanol to obtain an absorbance ranging from 149 (0.70 ± 0.2) to 734 nm (wavelength of maximum absorption) in a UV spectrophotometer. The first absorption is measured is ethanol which is white then an ABTS radical being measured time zero (T0) and A734. Then the last 6 minutes is measured by diluting 20 µl of DMSO in 2 mL of ABTS radical time of six minutes. For samples prepared from the extracts 20 mg of sample is diluted in 10 ml of DMSO then add 20 ml of diluting the sample in 2 ml of ABTS radical was generated by absorbance A734 was determined continuously 6 as minutes after dilution.

2.11. Content of Total Phenolic

For the test was used 2.5 ml of phenol reagent of Folin-Ciocalteu and 5 ml of Na_2CO_3 solution with a concentration of 20% is used, it was added to 0.5 ml aqueous solution at concentration 20 mg in 10mL of DMSO. The mixture remained 1 hour at 50˚C before measuring the absorbance at 750 nm. It was measured with a spectrophotometer of UV. The absorption of the developed blue color is measured at 750 nm and the total phenols content was determined from the calibration curve of standard solutions of gallic acid (1 - 20 mg/l) and expressed as mg gallic acid equivalents (GAE).

2.12. Statistical Analysis

Each test was performed in triplicate and the mean values were calculated. The data were reported as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Chemical Composition of the Extracts and Essential Oil

In the Chromatogram analysis of aqueous and ethanolic extracts of leaves (**Figure 1** and **Figure 2**) was identified as the compound rutin (quercetin-3-rutino-side). The aqueous extract showed a concentration of 12.56 mg/g of rutin and the ethanolic extract 11.66 mg/g of rutin, a very similar concentration of the extracts. Rutin is found in many plants of the family Fabaceae [21] [22], composed much appreciated by the pharmaceutical and cosmetic industries. Promising in the production of drugs to combat aging and degenerative diseases [23] and its main vasodilator function and antioxidant activity [24].

The essential oil chemical analysis by GC/MS, fourteen compounds were identified, the B germacrene the major compound with 39.28% of the total oil

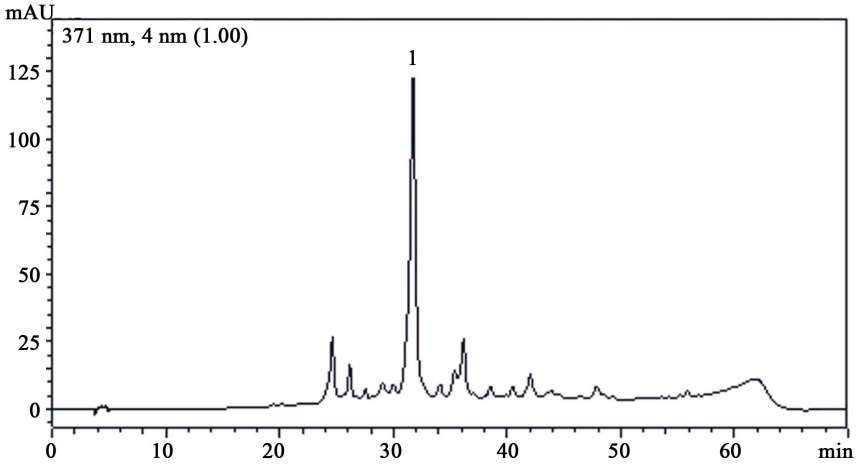

Figure 1. Chromatogram representing the wavelength of 371 nm, the quantification of rutin in the ethanolic extract of the leaves of the plant *Myrocarpus frondosus* Allemão by HPLC-DAD. Peak: 1—Rutin.

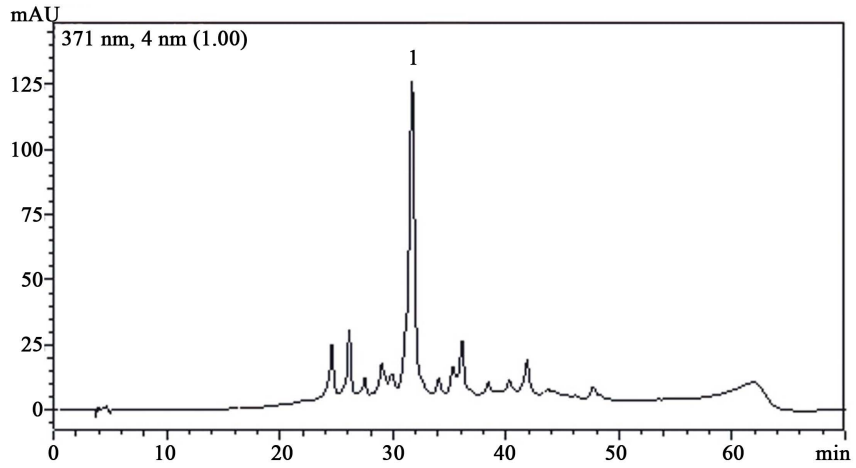

Figure 2. Chromatogram representing the wavelength of 371 nm, the quantification of rutin in the aqueous extract from the leaves *Myrocarpus frondosus* Allemão by HPLC-DAD. Peak: 1—Rutin.

compounds, followed by beta-copaene, beta-pinene, and Caryophyllene (**Table 1**) relating to the peaks compounds were represented in the Chromatogram 2 (**Figure 3**). The germacrene B, coapene beta, beta-pinene and Caryophyllene were classified as terpenes and essential oils were found in several families. Terpenes have a capacity of elimination of free radicals, thus gives them the high antioxidant capacity, moreover, has demonstrated a broad spectrum of antimicrobial activity against fungi and bacterial isolates [25] [26].

3.2. Antimicrobial Activity of Extracts, Essential Oil, Rutin and Trans-Caryophyllene

The results of the broth susceptibility test (**Table 2**) to determine the minimal inhibitory concentration (MIC), all compounds showed inhibition at concentrations of 1 mg/ml to 0.25 mg/ml for all the bacteria tested except for the essential oil and trans-Caryophyllene the concentration 0.25 mg/ml for *Staphylococcus*

Table 1. Essential oil chemicals of *Myrocarpus frondosus* Allemão plant performed by GC/MS.

Peak	Compound Name	Área%	R. Time
1	alpha-Pinene	1.01	4.981
2	Beta-Phellandrene	0.83	5.574
3	Beta-Pinene	11.03	5.641
4	Gamma-Elemene	0.56	11.008
5	Copaene	1.13	11.559
6	(-)beta-Bourbonene	0.86	11.700
7	Beta-Elemene	1.12	11.743
8	Caryophyllene	7.51	12.175
9	Humulene	1.57	12.614
10	Beta-Copaene	24.51	12.963
11	Germacrene B	39.28	13.166
12	Delta-Cadinene	1.23	13.432
13	Gamma-Muurolene	1.28	14.114
14	Spathulenol	7.03	14.160
15	ND	1.05	14.246
		100.00	

Table 2. Broth microdilution test of gram-positive and gram-negative front of the extracts, essential oil, rutin and trans-caryophillene.

Strains standards samples	Concentration (mg/mL)	E.c		S.a		L.m		S.t	
		MIC	CBM	MIC	CBM	MIC	CBM	MIC	CBM
EAF	1.0	−	+	−	−	−	−	−	−
	0.5	−	+	−	+	−	+	−	+
	0.25	−	+	−	+	−	+	−	+
EEF	1.0	−	+	−	+	−	+	−	+
	0.5	−	+	−	+	−	+	−	+
	0.25	−	+	−	+	−	+	−	+
RU	1.0	−	+	−	+	−	−	−	+
	0.5	−	+	−	+	−	+	−	+
	0.25	−	+	−	+	−	+	−	+
OE	1.0	−	−	−	−	−	−	−	−
	0.5	−	+	−	+	−	+	−	+
	0.25	−	+	+	+	−	+	−	+
TC	1.0	−	+	−	+	−	−	−	+
	0.5	−	+	−	+	−	+	−	+
	0.25	−	+	+	+	−	+	−	+

Inhibited growth (−); bacterial growth (+); MIC: Minimum Inhibitory Concentration; CBM: Minimum Bactericidal Concentration; E.c: *Escherichia coli*; S.a: *Staphylococcus aureus*; L.m: *Listeria monocytogenes*; S.t: *Salmonela thymurium*; EAF: aqueous extract of leaves; EEF: ethanolic extract of leaves; RU: Rutin; OE: Óil essential; TC: *Trans-cariofillene*.

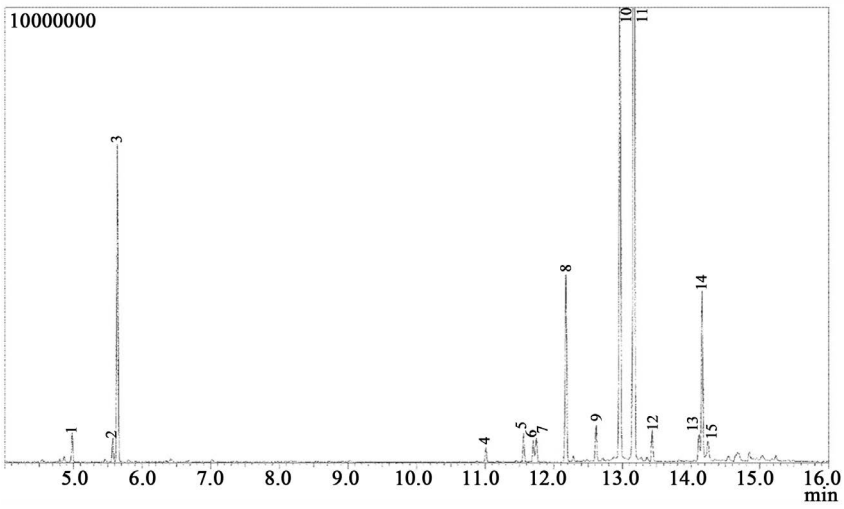

Figure 3. Chromatogram representing the peaks relating to the essential oil compounds from plant seeds *Myrocarpus frondosus* Allemão by GC/MS.

aureus. The compounds found in the essential oil are classified as terpenes, and these are recognized by the antibacterial and antioxidant activity [28] and compound rutin present in the extracts are known for their antioxidant activity. So because there are no studies in the literature reporting antimicrobial activity of plant *M. frondosus* against these bacteria.

The compounds found in the essential oil are classified as terpenes, and these are recognized by the antibacterial and antioxidant activity [27]. So far, there are no studies in the literature reporting antimicrobial activity of plant *M. frondosus* against these bacteria.

The Minimum Bactericidal Concentration (CMB) is defined as the lowest concentration of oil and/or extract resulting in the death of 99.9% of the inoculum. The results of CMB demonstrated that the essential oil had bactericidal activity at a concentration of 1mg/ml for all bacteria tested and trans-caryophyllene at the same concentration only for *Listeria monocytogenes.*

The 10% DMSO used as a control, there was growth of microorganism, thus confirming that the diluent does not interfere with the tests.

3.3. Phenolic Content Total (TPC) and Antioxidant Activity

In **Table 3**, can be observed the presence of phenolic compounds in ethanolic extracts of leaves in a concentration greater than the aqueous. But the essential oil concentration was lower. The concentration of phenolic compounds found in species of the same family may vary due to the extraction method, environmental stress, local collection period and the studied plant [21] [22] [29] [30] [31]. These phenolic compounds are effective as secondary metabolites and free radical scavengers and antioxidants [32].

The oil tested the antioxidant activity, has a capacity of inhibition of 40.92% (**Figure 4**), this can be explained by the presence of terpenes, these being responsible for the antioxidant activity [33]. In extracts, inhibition was lower compared to the essential oil (**Figure 4**). The antioxidant activity of the extracts

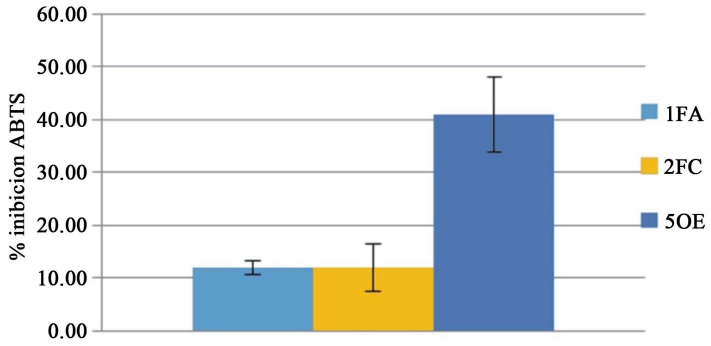

Figure 4. Graph representing the antioxidant activity by ABTS method of leaf]extracts and essential oil from the seeds of *Myrocarpus frondosus* Allemão plant. Where: 1FA: Aqueous extract of leaves; 2FC: Ethanolic extract of leaves; 5OE: Essential oil from the seeds.

Table 3. Quantification of phenolic compounds from aqueous and ethanolic extracts of leaves and essential oil from plant seeds *Myrocarpus frondosus* Allemão.

	Galic acid equivalents mg/L	Weight of oil (mg/L)	TOTAL PHENOLIC CONTENT (TPC%)
	C GAE		TPC %
1FA	198.979	2000	9.949
2FE	254.478	2000	12.724
5OE	120.221	2000	6.011

Where: FA: Aqueous extract of leaves; FE: Ethanolic extract of leaves; OE: Essential oil from the seeds.

may be related to the presence of rutin, one flavonoid, recognized for antioxidant activity and found in different concentrations in plants of the same family of *M. frondosus* [21] [22] [31]. The antioxidant activity of an extract or essential oil may vary depending on the antioxidant performance, the pro-oxidants, and the concentration of the mixture of chemical compounds [34].

4. Conclusion

The methodology used in this study allowed the identification of rutin in the extracts of leaves and germacrene B in the essential oil as major compounds. The results of this study clearly show that the essential oil had a *Myrocarpus frondosus* Allemão antioxidant activity in relation to the extracts that can be useful both in the pharmaceutical as in the food industry. In the antimicrobial activity, both as essential oil extracts appear to be promising, against Gram positive bacteria and Gram negative. However, additional studies were needed to isolate and identify active compounds, and to understand the mechanism of action as pharmacological agents *in vivo* studies.

Acknowledgements

We thank the laboratories LPPN, Food Microbiology, UPV/EHU, the Federal University of Pelotas and CAPES financial support.

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