

Inhibition of Drug-Sensitive and Drug-Resistant Mycobacterium Tuberculosis Strains by Essential Oil from *Croton Argyrophyloides* Mull. Arg.

ORIGINAL

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Abstract

Objective: To assess in vitro effects of essential oil extracted from *Croton argyrophyloides* against clinical and standard strains of Mycobacterium tuberculosis, comparing its resistance and sensitivity profiles to drugs commonly used in therapy.

Method: In the present study, chemical composition and antimycobacterial activity of essential oils extracted from aerial parts of *Croton argyrophyloides* collected in Curitiba district, Sergipe, Brazil were analysed. The oil was studied by GC and GC-MS and its antimycobacterial activity (MIC) was tested against 49 clinical and standard covers H₃₇Rv using the REMA method. To access sensitivity to isoniazid, rifampicin, ethambutol and streptomycin it was used proportions indirect test method.

Results: Twenty-seven chemicals were identified, totalizing 95.98% of the composition of essential oil. The oil presented good antibacterial activity (MIC = 97 to 195 g / ml) against strains of Mycobacterium tuberculosis H₃₇Rv and pattern one.

Conclusion: On pharmacological activities of these species confirmed in vitro scientific support for its use in traditional herbal preparations.

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Keywords

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Introduction

Mycobacteria spp. have increased their virulence and tuberculosis (TB) has become the most lethal infection in the world nowadays. Between 1980 and 2005, 90 million cases of TB worldwide were reported to the World Health Organization (WHO). One of the biggest problems about treatment and controlling of TB infection is changes in strains behavior against several drugs due to anti-TB drugs resistance induced by HIV epidemic [1].

In Brazil, many communities use medicinal plants as alternative treatment of infectious diseases, including tuberculosis [2]. Cruz [3] conducted an ethnobotanical study in Curituba village (Sergipe-Brazil), about medicinal plants used for dermatophytosis, tuberculosis and others, including *Croton argyrophyloides* Mull. Arg., locally known as "sacatinga".

Croton genus is one of the largest in *Euphorbiaceae* family, ranging about 800 species. Brazil has registered about 300 species. Widely distributed in northeast of Brazil, especially in sandy ground, *Croton spp.* is used in many diseases treatments among people living in these areas [2, 4].

This study aims to assess in vitro effects of essential oil extracted from *Croton argyrophyloides* against clinical and standard strains of *Mycobacterium tuberculosis*, comparing its resistance and sensitivity profiles to drugs commonly used in therapy.

Methods

Plant material

Plant selection was based on ethnobotanical study of popular uses of medicinal plants conducted by Cruz [3]. Botanical material was collected on November, 11th, 2009, georeferenced as (S 09°39'40.0", W 037°55'16.3"; Altitude: 205 meters). Plant material was collected, then evaluated and identified by Ms. Gilvane Viana Souza (Botanical Department of Federal University of Sergipe, UFS).

A voucher specimen selected plant was deposited at the Herbarium ASE under the register number: ASE 13161.

Essential oil

The essential oil was obtained by hydrodistillation (for 3 h) of *C. argyrophyloides* fresh leaves in a Clevenger-type apparatus, until no more condensing oil could be seen. The essential oil was separated from the aqueous solution (hydrolate), dried with anhydrous Na₂SO₄ (yield 0.5% v/w), transferred to an amber glass flask, and kept at temperature of 14 °F (-10 °C) until used.

Gas chromatography – mass spectrometry (GC-MSms)

Oil sample analysis was performed on a Shimadzu QP5050A gas chromatograph (Shimadzu Corporation, Kyoto, Japan), interfaced to a mass spectrometer (GC-MS). The following conditions were used: DB5 column (30 cm x 0.25 mm i.d., 5% phenylmethylpolysiloxane); helium (99.999%) carrier gas at a constant flow of 1.2 mL min⁻¹; 0.5 µL injection volume (in ethyl acetate); injector split ratio of 1:20; injector temperature 250 °C; electron impact mode at 70 eV; ion-source temperature 280 °C. The oven temperature was programmed from 80 °C (isothermal for 2 min), with an increase of 3 °C min⁻¹ to 180 °C, then 10 °C min⁻¹ to 300 °C, and 10 min isothermal at 300 °C. Mass spectra were taken at 70 eV, with a scan interval of 0.5 s, and considered ion fragments in the range 40 to 450 Da.

A mixture of linear hydrocarbons (C₉H₂₀–C₁₉H₄₀) was injected under the same conditions as samples, and identification of constituents performed by comparing the spectra obtained with those of the equipment's database (NIST 21 and NIST 107), and by using Kovats Index, calculated for each constituent as previously described [5].

Microorganisms

Forty-nine clinical strains of *M. tuberculosis* and standard strain H₃₇RV were tested. These strains were part of culture collections from Central Laboratory of Sergipe and Applied Microbiology Laboratory, Federal University of Sergipe.

Sensitivity and resistance profile of *M. tuberculosis* strains: the proportion method procedure

The proportion method was performed as described by National Committee for Clinical Laboratory Standards (NCCLS 2002). Antimycobacterial drugs were adjusted in the LJ medium to final concentrations of 0.2-1 µg/mL for isoniazid (INH), 40 µg/mL for rifampicin (RIF), 4 µg/mL for streptomycin (STR), and 2 µg/mL for ethambutol (ETM). One hundred microliter of prepared bacterial inoculum was inoculated on LJ medium, containing or not drug for test or as a control, followed by incubation at 37°C for 21-28 days. Resistance was defined as growth on drug containing tubes greater than 1% of the growth of drug free control medium for INH, RIF, ETM, and 10% for STR [6,7].

Antimycobacterial activity of essential oil

The method used for antimycobacterial activity determination was [8]. The screening assay was, in brief, accomplished in microplates (96 wells) using resazurin as indicator of cellular viability. The concentrations for essential oil ranged from 125.000µg/mL até 97µg/mL. Briefly, to prepare inoculums, bacterial suspension adjusted to equal the density of a 1.0 McFarland standard was diluted 1:25 in medium 7H9 enriched with 10% OADC. To each well was added 100 µl of inoculums and 100 µl concentrations for essential oil. The plates were sealed, placed in plastic bags and incubated at 37°C for seven days in normal atmosphere. After seven days of incubation, 30 µl of resazurin solution was added to each well, incubated at 37°C, and assumed for color development. A change from blue to pink in-

dicates reductions of resazurin and therefore bacterial growth. The MIC was defined as the lowest essential oil concentration that prevented this color change.

Results

Chemical compounds identified, their percentages and retention indices of *C. argyrophyloides* Mull. Arg. essential oil are listed in Table 01. Out of 30 compounds found, 27 were identified by gas chromatography, which confirmed a high content of terpenes. It was found a major percentage of metabolites classified as monoterpenes (48.22%) and sesquiterpenes (47.76%). The major monoterpenes were: myrcene 7.92%, α-pinene 7.15%, the β-phellandrene 5.43%, 1,8-cineole 5.23% and δ-3-Carene 4.27%.

About sesquiterpenes, Bicyclogermacrene showed the highest concentration totalizing 12.99% of all chemicals identified, followed by spathulenol (11.33%) and β-caryophyllene (9.76%).

Three metabolites of essential oil were detected by mass spectrometry, but were not identified by gas chromatography, corresponding to 5.57% of chemical composition of the sample. (Table 1)

The essential oil of *C. argyrophyloides* was tested against 49 clinical strains and one standard strain (H₃₇Rv). Nineteen clinical strains belonged to Bank of Strains of Applied Microbiology Laboratory, Federal University of Sergipe (AML / UFS) and 30 clinical strains and the standard strain belonged to the Central Laboratory of Sergipe.

Results obtained in tests of Minimum Inhibitory Concentration-MIC with *C. argyrophyloides* Mull. Arg. essential oil and susceptibility profile of *M. tuberculosis* strains against rifampicin (RIF), isoniazid (INH), ethambutol (ETM) and streptomycin (STR) are shown in Table 2

Minimum inhibitory concentrations of essential oil tested by REMA method standardized by Palomino [8] ranged between 97 and 195 µg/mL for clinical

Table 1. Essential oil composition (%) of *Croton argyrophylloides* Mull. Arg.

Peak	RT (min)	Compound	(%)	RRI exp. ^a	RRI lit. ^b
1	6.583	α-Thujeno	2.35	917	924
2	6.808	α-Pineno	7.15	925	932
3	8.058	Sabineno	2.47	968	969
4	8.217	β-Pineno	3.18	974	974
5	8.608	Mirceno	7.92	988	988
6	9.258	δ-3-Careno	4.27	1008	1008
7	9.833	p-Cimeno	0.49	1024	1020
8	9.992	Limoneno	2.30	1029	1024
9	10.050	β-Felandreno	5.43	1030	1025
10	10.108	1,8-Cineol	5.23	1032	1026
11	10.625	(E) β-Ocimeno	0.85	1046	1044
12	11.042	γ-Terpineno	0.64	1058	1054
13	12.017	Terpinoleno	1.10	1085	1086
14	12.583	Linalol	1.81	1100	1095
15	15.567	Terpinen-4-ol	1.31	1181	1174
16	16.117	α-Terpineol	0.85	1196	1186
17	21.125	δ-Elemeno	0.65	1335	1335
18	21.533	Acetato de α-Terpineno	0.22	1347	1346
19	23.000	β-Elemeno	4.05	1390	1389
20	24.017	β-Cariofileno	9.76	1420	1417
21	25.208	α-Humuleno	1.53	1456	1452
22	26.508	Biciclogermacreno	12.99	1496	1500
23	26.900	Germacreno A	0.93	1508	1508
24	27.958	α-Calacoreno	0.58	1542	1544
25	28.517	Germacreno B	2.77	1560	1559
26	28.617	Não identificado	4.97	1563	-
27	29.075	Spathulenol	11.33	1578	1577
28	29.250	Óxido de Cariofileno	2.30	1584	1582
29	29.942	Não identificado	0.11	1606	-
30	30.217	Não identificado	0.49	1615	-

^a: RRI = relative retention index using the Van den Dool equation. 1963 [9].

^b: According to Adams, 1995 [5].

Table 2. MIC (µg/mL) of essential oil from *C. argyrophylloides* Mull. Arg. and sensibility profile of *M. tuberculosis* strains to anti-TB drugs

Strains	MIC ^a of essential oil (µg/mL)	RIF ^b	INH ^c	ETM ^d	STR ^e
HRV37	97	S	S	S	S
13; 21; 34; 37; 43; 133; 594; 723; 825; 846; 869;870; 889; 1047; 1135; 1145; 1290; 1307; 1328; 1374; 050; 770; 2580; 2910; 4840; 4930; 5020; 5550; 6870; 7700; 8510; 9110; 9210; 10810; 12090; 12930; 14050;14880; 15060	97	S	S	S	S
658	195	R	R	R	S
725; 1317	195	S	R	R	S
810; 909; 948; 1322; 1343	97	S	S	S	R
1057	195	S	R	S	S
1224	195	R	R	S	S

^a: Minimal inhibitory concentration;

^b: Rifampicin; ^c: Isoniazid; ^d: Ethambutol; ^e: Streptomycin.

strains. Only five strains showed MIC equal to 195 µg/mL. All other strains including standard strain (H₃₇Rv) had MIC of 97 µg/mL.

Results of *M. tuberculosis* susceptibility analysis from gold standard chemotherapy recommended by Health Ministry showed that out of 49 strains tested, 10 were resistant to one chemotherapeutic at least. Strains resistant to anti-tuberculosis drugs revealed a prevalence of 20% (**Table 2**).

Correlation between strains resistant to anti-tuberculosis drugs and MIC values found for essential oil tested showed that four clinical strains which were resistant to more than one of the antibiotics tested, and two clinical strains which were considered multiresistant showed MIC values for *C. argyrophylloides* Mull. Arg. essential oil equal to 195 µg/mL. Additionally, in all strains that showed resistance to RIF, INH and/or ETM, MIC values for *C.*

argyrophyloides Mull. Arg. essential oil was equal to 195 µg/mL. In all strains resistant to STR, MIC values for *C. argyrophyloides* Mull. Arg. essential oil was equal to 97 µg/mL.

Discussion

Use of medicinal plants in curing TB-related symptoms is reported, especially, in South American and African regions, where health services access is precarious and TB rates are more significant among people living under low social and economical conditions. Researches for new anti-TB drugs include screening bioactive compounds of vegetable sources [1, 10]. Thus, testing *C. argyrophyloides* Mull. Arg. essential oil against clinical and standard strains of *M. tuberculosis*, which tuberculosis treatment regimen drugs sensitivity profile has shown resistance or multiresistance, was useful.

Most antibiotics used in experiments of this study are part of Schedule I to treat tuberculosis [11]. In isolation, Streptomycin and Isoniazid were less effective, both presenting five strains resistant. Studies [12, 13, 14, 15, 16] confirm isoniazid to be less effective against *M. tuberculosis*. By these studies, STR was more effective than INH. Differently, in our data both had the same strains resistance profile. For Ethambutol, three strains were resistant. The most effective antibiotic was Rifampicin, which only two samples of *M. tuberculosis* were resistant; these strains were concomitantly resistant to rifampin and isoniazid, classifying them as multidrug resistant strains, according to literature [14]. Evaluating this study results, one can observe that there is a change in resistance and multidrug resistance profile to medication regimens used. Thus, research for active and less toxic substances against mycobacteria has motivated to find solutions for tuberculosis treatment since ancient times [17].

C. argyrophyloides Mull. Arg. traditionally is traditionally used by district of Curituba communities, in Sergipe, Brazil, for TB-related symptoms

treatment. Some species of *Croton* gender, *C. pseudopulchellus* and *C. sylvaticus*, have already confirmed their antimycobacterial activity [9,18]. These studies corroborate popular knowledge of its use in TB-therapy and demonstrate the importance of this kind of drug discovery [19, 20, 21, 22, 23, 24].

Our data has shown that all 49 clinical strains and standard H₃₇Rv *M. tuberculosis* tested were sensitivity to essential oil extracted from fresh leaves of *C. argyrophyloides* Mull. Arg. with MIC values equal to 97 µg/mL (45 strains) and 195 µg/mL (05 strains). Noteworthy, all strains sensitive to antibiotics tested showed sensitivity to the essential oil at concentration of 97 µg/mL; in contrast, five strains that showed sensitivity at higher concentration (195 µg/mL), were resistant to isoniazid. The low MIC values of essential oil of *C. argyrophyloides* Mull. Arg. can be considered promising when compared to MIC values found by some authors, as compared to other products derived from plants. Allied to this fact, it is important to remember that most researches are carried out exclusively with standard strains, without clinical strains as we did, frequently subjected to constant change and therefore more resistant.

Study [25] tested extracts from five plants (*Marrubium vulgare*, *Mentha spicata*, *Artemisia ludoviciana*, *Chaenopodium ambrosioides* and *Flourens cernua*), compared to *M. tuberculosis* standard strains and found MIC values equal to 400 µg/mL. Study [26] found antimycobacterial action in ten plants from South Africa; some of these plants has action set at 195 µg/mL.

In Mexico, several studies have demonstrated potential of regional plants against *M. tuberculosis* in vitro. Extract taken from *Buddleja cordata* is active at concentration of 64 µg/mL [28, 29] uses extracts from 48 plants originated in South America to standard strains H₃₇Rv and only seven from them shows action at concentration of 100 µg/mL.

MICs values found in this study may be associated to the interaction of oil with *M. tuberculosis* cell

wall, since it is rich in high molecular weight lipid, facilitating the contact with nonpolar substances [30].

Mono and sesquiterpenes percentage we found were similar to those reported by [4, 20], when identifying chemical compounds of *C. argyrophyloides* Mull. Arg. essential oil collected in Viçosa, Ceará, Northeastern Brazil. Differences in percentage composition of essential oil can exist and are due to temperature and season of the region where he collected the plant material, location, storage, methods of oil extraction and others. However, presence of these substances in relevant different percentage can mean genetic inheritance of the [31].

In spite of previous studies of *C. argyrophyloides* Mull Arg. essential oil performance, the need for checking chemical composition is justified, once his action is related to existing concentration of each substance and possibly with the synergistic action of them [32].

Despite antimycobacterial potential *in vitro* of *C. argyrophyloides* Mull Arg. essential oil evidenced by MICs values, it is important that studies on mechanisms of action and activity *in vivo* are carried out to confirm its effectiveness in treating the symptoms of TB.

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