

Evaluation of *Maytenus aquifolia* Mart. and *Maytenus ilicifolia* Mart. chemotypes for tannins, total phenols and triterpenes

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ABSTRACT: *Maytenus aquifolia* and *Maytenus ilicifolia* are medicinal species with validated antiulcerogenic effects, which have configured demand in Brazil as source of active principle in phytotherapeutic products. In the present work, different accessions of these two species were evaluated for triterpene, total phenolic and tannin contents and it was verified that there is no direct correlation among yields of those chemical markers and the morphological characteristics observed.

Key words: *Maytenus aquifolia*, *Maytenus ilicifolia*, Celastraceae, chemical race, triterpene, phenols, tannins.

RESUMO: Avaliação dos quimiotipos de *Maytenus aquifolia* Mart. e *Maytenus ilicifolia* Mart. em relação aos taninos, fenóis totais e triterpenos. *Maytenus aquifolia* e *Maytenus ilicifolia* são plantas medicinais com comprovados efeitos anti-ulcerogênicos, os quais têm estabelecida demanda no Brasil como fonte de princípios ativos em produtos fitoterápicos. No presente trabalho, diferentes acessos destas duas espécies foram avaliadas em relação aos teores de triterpenos, fenólicos totais e taninos. Contudo, foi verificado que não há correlação direta entre a produção daqueles marcadores químicos e as características morfológicas observadas.

Palavras-chave: *Maytenus aquifolia*, *Maytenus ilicifolia*, Celastraceae, grupo químico, triterpeno, fenóis, taninos.

INTRODUCTION

Maytenus aquifolia and *Maytenus ilicifolia* belong to the Celastraceae family and are popularly known in Brazil as "Espinheira Santa". The antiulcerogenic effect of leaf aqueous extracts from these species has been confirmed by Souza-Formigoni (1991), and it is related to total phenols, more specifically the tannins, to triterpenes (Pereira et al., 1993; Corsino et al., 2000) and also to flavonoid glycosides (Vilegas et al., 1999; Leite et al., 2001; Corsino et al., 2003). These species have been validated by clinical and toxicological assays and they have been amply used in Brazil.

Since 1988 the University of Ribeirão Preto - UNAERP holds a germoplasm bank with accessions proceeding from seeds and wild plantlets of *M. aquifolia* and *M. ilicifolia* from distinct regions of three Brazilian states, Paraná, Santa Catarina and Rio Grande do Sul. In this bank, morphologically diversified plant material presenting specific inter and intra differences is preserved. Pereira et al., (1994b) reported

that seeds of *M. aquifolia* plants present considerable genetic variability showing varied architecture, yields and profile of secondary metabolites accumulated. The different chemotypes of *M. aquifolia* and *M. ilicifolia* found in the germoplasm bank at UNAERP are examples of the genetic patrimony of those species and evolutionary change during the adaptive processes to different environmental conditions, and represent valuable resources to be studied and preserved.

The objective of the present work was to determine yields of tannins, total phenols and triterpenes in diverse genotypes of *M. aquifolia* and *M. ilicifolia* and check possible correlation among contents of active compounds and morphological differences found in these species.

MATERIAL AND METHOD

Plant material

Ten-year-old *M. aquifolia* Mart. and *M.*

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ilicifolia Mart. (Celastraceae) plants from germinated seeds, maintained at UNAERP germoplasm bank, were collected in September 1997. Leaves harvested from 7 different accesses of *M. ilicifolia* and 4 accesses of *M. aquifolia* were stabilized and dried in air conditioned room at $25 \pm 2^\circ\text{C}$. A voucher specimen has been deposited in the UNAERP (Universidade de Ribeirão Preto) herbarium under number HPM 054 (*Maytenus ilicifolia*) and HPM 268 (*Maytenus aquifolia*).

The different genotypes were identified using numbers from 1 to 11 for both species of *Maytenus*. Numbers from 1 to 7 were attributed to *M. ilicifolia* genotypes that presented differences in number of thorns on the border of the leaves and leaf size. Numbers from 8 to 11 were used for *M. aquifolia* genotypes that showed discrepancy in the size of leaves of the same plant.

Standard compounds

Standards were isolated from hexanic extract of *M. aquifolia* leaves via chromatographic methods. The isolated compounds structures were proposed comparing with spectral data, ^1H and ^{13}C NMR, previously reported (Anjaneyulu, 1980; Mahato, 1994) and with authentic standards.

Quantitative analysis

Phenols

Powdered *M. ilicifolia* and *M. aquifolia* leaves (200 mg) of each genotype were extracted with boiling $\text{MeOH}/\text{H}_2\text{O}$ 1:1. The dosage of phenols in the leaf extracts was carried out using the official AOAC method adapted for plant material analysis (AOAC, 1984, Velozo, 1988). Total phenol concentration was determined using a UV spectrophotometer set at 730 nm.

Tannins

Contents of total tannins in *M. ilicifolia* and *M. aquifolia* leaf extracts were determined using the Hide powder method described in the Farmacopéia Brasileira (4th. Ed. 1996). Absorbancy was measured at 715 nm using pyrogallol as standard.

Chromatographic analysis of triterpenes

Powdered *M. ilicifolia* and *M. aquifolia* leaves (1 g) of all the genotypes were extracted separately in ethyl acetate. Extracts were then dechlorophyllated using 8 g florisil™ (Aldrich 100-200 mesh). After solvent elimination, residue was dissolved in chloroform and samples (1 μL) were chromatographed. Gas chromatography was carried out using a Varian Star 3400 chromatograph; equipped with split-splitless injector, flame ionization detector and a Varian 4400 integrator. The chromatographic separation was achieved with a DB1 capillary column (methylpolysiloxane 30 m x 0,25 mm x 0,1 μm

thickness). Nitrogen was the carrier gas at a flow rate of 0,8 mL min^{-1} . Injector and detector temperatures were 250°C and 300°C , respectively. Samples (1 μL) were injected in the split mode at a split ratio of 1:20. Column temperature was set at 240°C , followed by further heating to 300°C maintaining the final temperature for 4 min.

The intraassay precision was evaluated using 7 samples of *M. aquifolia* (genotype nº 8). Linearity of the triterpene analysis method was obtained using friedelin solution within the range of 10-1000 $\mu\text{g mL}^{-1}$ as external standard.

Structural identification of isolated compounds was performed through comparison of spectral ^1H , ^{13}C NMR data with that reported in the literature (Anjaneyulu, 1980; Mahato, 1994).

The triterpene analysis showed linearity within the range of 10-100 $\mu\text{g mL}^{-1}$ with the coefficient of correlation greater than 0,99.

RESULT AND DISCUSSION

The extraction method employed presented relative standard deviation inferior to 10%, assuring reproducibility of obtained results.

The quantification limit (lowest concentration detected by proposed method with intraassay precision with variation coefficients inferior to 20%) was 10 $\mu\text{g/mL}^{-1}$.

The substances analyzed are correlated to the pharmacological activity of this species. Triterpenes, tanins and phenolic compounds considered cicatricial agents of gastric ulcers (Vervloet, 2003).

Figure 1 shows the triterpene analysis chromatogram profile with efficient resolution of triterpenes, no interferences was observed co-eluting with friedelin or friedelan-3-ol compounds.

Yields of secondary metabolites analyzed varied significantly among the genotypes of the same species and between the two studied species. *M. aquifolia* showed higher levels of triterpenes compared to *M. ilicifolia* (Table 1), although contents of tannins and total phenols found in *M. ilicifolia*, i.e., yields of phenolic compounds in *M. ilicifolia* were three fold higher (Table 1). According to McKey et al., (1978) phenolic compounds are of great importance for plants because they confer resistance against microorganisms and defense against herbivores. It was observed that cultured *M. ilicifolia* plants are less susceptible to microbial attack than *M. aquifolia* suggesting that high levels of phenolics help the plant defense.

Overall results indicate that it is necessary to select superior chemotypes for the multiplication process of plant material to be used as crude material in the production of phytotherapies.

The popular name for both *Maytenus* species

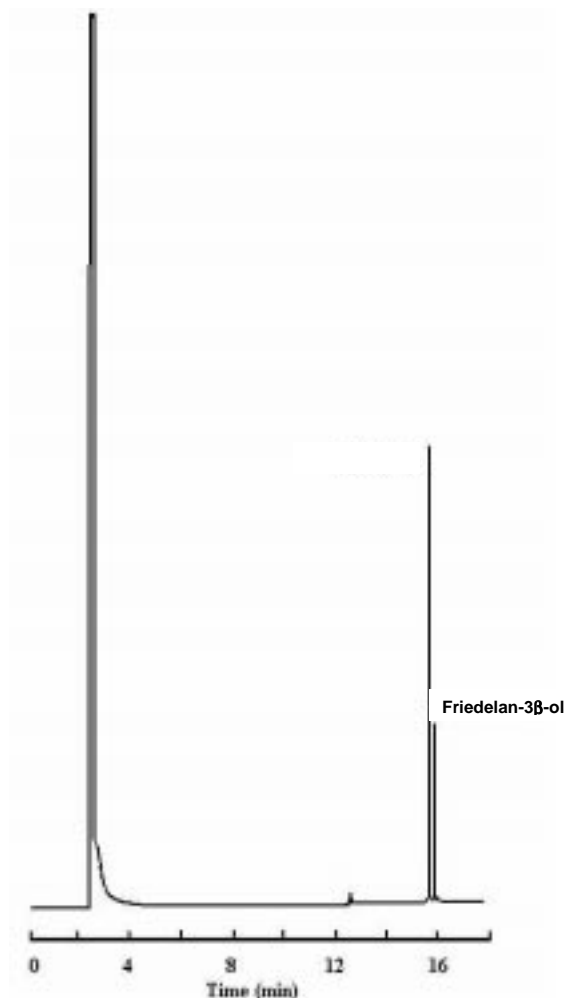


FIGURE 1. Chromatogram of analysis of triterpenes in *M. aquifolia*

is espinheira santa that means "holy thorn". They were named due to the abundance of thorns on the border of their leaves. However, leaves of many accesses of *M. ilicifolia* present reduced number or almost no thorns on the borders and sometimes for this reason plants are commercially rejected since people suspect about the authenticity and therapeutic value of such species. Table 2 shows that the accesses nº 5, 6 and 7 of *M. ilicifolia* presented reduced number of thorns on the border of the leaf, although that morphological characteristic can not be correlated to yields of any class of secondary metabolite analyzed. The same occurred with the accesses of *M. aquifolia* that despite of showing differences on foliar area did not present discrepancy on contents of the natural product accumulated. Obtained results showed that there is no correlation between number of thorns and yields of bioactive molecule. The profusion or scarcity of thorns on the border of the leaf does not provide authenticity to the species.

Most of the medicinal plants are still grown as wild plant with a wide range of genetic variability. Thus, the selection of suitable genotypes for desired traits can be achieved relatively easily. Selection for suitable cultivars must take into account both the biomass yield and the phytochemical contents. The heritability of these two different and complex characteristics is generally different. The biosynthesis of chemicals is generally a dominant factor however, the integration of both the yield of biomass and the yield of phytochemical compounds result in a much lower heritability rate (Palevitch, 1991).

Yields of secondary metabolites produced in plants are not stable and are not homogeneously distributed in the parts of the plant. Several conditions influence the accumulation of bioactive molecules in

TABLE 1. Yields (%) of triterpenes, phenols and tannins in *M. aquifolia* and *M. ilicifolia*

Species (access nº)	Friedelan-3-ol (%)	Friedelin (%)	Total phenols (%)	Total tannins (%)
M. ilicifolia				
(1)	0.45±0.01 a	0.20±0.01 a	14.07±0.50 c	3.78±0.18 c
(2)	0.33±0.02 bc	0.11±0.03 cd	17.75±0.97 b	4.78±0.15 b
(3)	0.41±0.01 ab	0.14±0.01 b	16.78±0.49 b	3.84±0.25 c
(4)	0.42±0.03 ab	0.13±0.02 bc	17.91±0.39 b	4.79±0.04 b
(5)	0.28±0.01 c	0.11±0.01 cd	17.27±0.36 b	5.26±0.19 ab
(6)	0.40±0.02 ab	0.10±0.01 d	17.34±0.42 b	5.48±0.11 a
(7)	0.41±0.01 ab	0.12±0.01 bc	20.00±0.31 a	5.87±0.25 a
M. aquifolia				
(8)	0.61±0.02 C	0.16±0.01 B	5.85±0.55 AB	1.21±0.10 AB
(9)	0.81±0.03 A	0.20±0.02 A	6.28±0.52 A	1.45±0.08 A
(10)	0.71±0.03 AC	0.21±0.04 A	7.53±0.96 A	1.53±0.06 A
(11)	0.75±0.01 AB	0.20±0.01 A	4.23±0.43 B	0.83±0.06 B

Ob. Treatment means within rows followed by the same letters are not significantly different ($p < 0.05$). Capital letters for *M. aquifolia* and small letters for *M. ilicifolia*

TABLE 2. Morphological data of *M. ilicifolia* and *M. aquifolia* leaves.

Species (access nº)	Nº of thorns on leaves	leaf height (cm)	leaf width (cm)
M. ilicifolia			
(1)	9	3.0	1.1
(2)	11	3.1	1.2
(3)	11	3.5	2.1
(4)	15	5.5	2.2
(5)	1	4.2	1.3
(6)	4	4.1	1.5
(7)	3	3.6	1.5
M. aquifolia			
(8)	13	6.1	2.0
(9)	17	7.3	3.0
(10)	19	6.4	2.5
(11)	19	5.1	2.0

plants, either genetic factors or even external factors as temperature, rain, wind, latitude, altitude and soil. Also, technical factors as period and type of harvest, cultural tracts, propagation methods and post harvesting processing (Marshall & Jain, 1968; Sultan, 1987; Gillespie & Turelli, 1989; Via, 1987; Goldstein & Holsinger, 1992; Jarvis, 1995; Hayashi et al., 1996; Passreiter & Aldana, 1998; Buter et al., 1998).

According to the review published by Ferreira & Duke (1997) the selection of high-yield natural strains, varieties or chemotypes has been the method generally used in obtaining plant with high yields of natural products. These authors have reported that there was almost five-fold difference in total foliar taxane content of 57 cultivars of *Taxus*, and chemotypes of *Podophyllum peltatum* from North exhibited a large range in podophyllotoxin content.

The experience acquired in the last ten years with *Maytenus* species has showed that plants from seeds show great morphological variability and accumulate different contents of secondary metabolites, although this experience was not divulged in the present work. These findings reinforce the opinion that plant propagation techniques via stalks or micropropagation (protocol already established by Pereira et al., 1994(a); Pereira et al., 1995) are the most appropriate for the multiplication of *Maytenus*, since they allow the reproduction of elite chemotypes that show superior productivity of active compounds. From the 11 accesses investigated in this work we selected the accesses nº4 of *M. ilicifolia* and nº9 of *M. aquifolia* as the most promising for large scale production.

After following the development of *Maytenus ilicifolia* and *M. aquifolia* for 10 years it was possible to conclude that *M. aquifolia* is better adapted to the Southeast and Western regions while *M. ilicifolia*

grows better in the South region of Brazil. *M. ilicifolia* plants cultured in our region showed a very slow growth.

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