Antioxidant and genotoxic properties of *Maytenus dasyclada*: a comparative study in relation to *Maytenus* reference species

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Received: October 10, 2013 – Accepted: December 4, 2013 – Distributed: May 31, 2015
(With 3 figures)

Abstract

In these work the in vitro antioxidant activity and the in vivo genotoxicity of *M. dasyclada* was compared to the reference species *M. aquifolium* and *M. ilicifolia*. *M. dasyclada* showed in vitro antioxidant activity comparable to *M. aquifolium* but lower than *M. ilicifolia*, being that a inverse Pearson correlation between DPPH IC_{50} values and total phenolic content was detected (–0.932). The carbonyl content of *M. dasyclada* and *M. aquifolium* extracts promoted a similar increase in protein oxidation in vivo, while *M. ilicifolia* no altered the carbonyl levels. The comet assay demonstrated that the three analyzed species promoted a low and similar level of genotoxicity; which is compatible with DNA damage induced by other medicinal plants and is partially recovered by a co-treatment with vitamin C. The data showed *M. dasyclada* as antioxidant activity in vitro, and that its genotoxic and pro-oxidant effects in vivo are comparable to the *Maytenus* reference species.

Keywords: Maytenus ilicifolia, *M. aquifolium*, *M. dasyclada*, comet assay, antioxidant activity, phenolic pompounds.

Propriedades antioxidantes e genotóxicas de *Maytenus dasyclada*: um estudo comparativo em relação às espécies de referência de *Maytenus*

Resumo

No presente trabalho a atividade antioxidante *in vitro* e a genotoxicidade *in vivo* de *M. dasyclada* foi comparada com as espécies de referência *M.aquifolium* e *M. ilicifolia*. *M. dasyclada* mostrou atividade antioxidante *in vitro* comparável a de *M. aquifolium* mas inferior a *M. ilicifolia*, sendo que foi detectada uma correlação de Pearson inversa entre os valores de IC_{50} por DPPH e o conteúdo fenólico total (–0,932). Em relação ao teor de carbonila, os extratos de *M. dasyclada* e *M. aquifolium* promoveram um aumento semelhante na oxidação das proteínas *in vivo*, ao passo que *Maytenus ilicifolia* não alterou os níveis de carbonila. O ensaio do cometa demonstrou que as três espécies analisadas promoveram um nível baixo e semelhante de genotoxicidade, o que é compatível com os danos no DNA induzidos por outras plantas medicinais e é parcialmente recuperada por um co-tratamento com a vitamina C. Os dados mostraram *M. dasyclada* com atividade antioxidante *in vitro*, e que os seus efeitos genotóxicos e pró-oxidantes *in vivo* são comparáveis às espécies de referência de *Maytenus*.

Palavras-chave: Maytenus ilicifolia, *M. aquifolium*, *M. dasyclada,* teste cometa, atividade antioxidante, substâncias fenólicas.

1. Introduction

Maytenus aquifolium Mart. (synonym *M. aquifolia*) and *M. ilicifolia* Mart. ex Reissek (synonym *M. muelleri*) (Celastraceae) are widely employed in Brazilian popular medicine, being known as 'espinheira-santa' or 'cancarosa'. Both are native plants, with natural occurrence in South of Brazil and are used in form of teas for stomach and ulcer illness treatment (Mariot and Barbieri, 2007). Souza-Formigoni et al. (1991) have demonstrated the antiulcerogenic property of these two species, which seems to be closely related to the presence of two classes of substances, namely, phenols and triterpenes (Mossi et al., 2009).

This increasing interest by natural antioxidants is related to their low toxicity as compared to synthetic antioxidants (Soares et al., 2008). In these sense, investigations about

the presence of new antioxidants in plants emerges as a natural alternative against the deleterious effects of reactive oxygen species (ROS) and free radicals (FR) (Galvão et al., 2008). Several works have already shown the antioxidant activity of *M. aquifolium* and *M. ilicifolia* (Corsino et al., 2003; Vellosa et al., 2007; Mariot and Barbieri, 2007; Pessuto et al., 2009), which probably is due to the action of polyphenols and flavonoids as the free radicals scavengers. The pharmacological properties of *M. ilicifolia* were reviewed in literature (Santos-Oliveira, 2009).

The production of ROS and FR can occur as consequence of exposition to toxic agents (radiation, antibiotics, several types of environmental pollutants, among others) or as result of normal metabolism in the living cells (Halliwell and Gutteridge, 2007). In situations where the ROS and FR production exceeds the defense capacity of the organism, a condition known as oxidative stress can occurs and provoke damages to important biomolecules, including DNA, proteins and membrane lipids, compromising cell general functions (Vargas et al., 1991).

Despite of the increasing in the use of medicinal plants in the last decade, the most of them was not sufficiently studied in relation to the quality, safety, efficiency patterns or even in relation to their cytotoxic and mutagenic potential (Bagatini et al., 2007). Currently, the application of *M. ilicifolia* as medicinal plant is allowed in Brazil, so that their register and commercialization in the phytomedicine don't requires the application of efficiency and safety tests (Brasil, 2011).

However, *Maytenus dasyclada* have been popularly used as teas without knowledge of their therapeutic properties and their toxic and mutagenic potential. There is no literature that describes the antioxidant activity of the specie in vivo. There is only one manuscript in the literature about chemical characterization and in vitro biological properties of this specie (Schwanz et al., 2013). These work identified the presence of quercetin and kaempherol in ethyl acetate and ethanolic extracts of *M. dasyclada*, being that both showed antioxidant and antigenotoxic activity in vitro. However, there is no literature describing biological properties of *M. dasyclada* in vivo.

Viewing expands the knowledge about *Maytenus* genus and their economic and/or medicinal potential, the objective of this study was compare the in vitro antioxidant activity and the in vivo genotoxicity of *M. dasyclada* with the reference species *M. aquifolium* and *M. ilicifolia*.

2. Material and Methods

The leaves of *Maytenus* species were collected in the northern Rio Grande do Sul, Brazil. The exsicatas were identified and deposited in the Herbarium Padre Balduino Rambo of the URI University, Erechim, RS, Brazil. The exsicatas were identified by the numbers HPBR 11,512 (*M. dasyclada*), HPBR 11,508 (*M. aquifolium*) and HPBR 11,502 (*M. ilicifolia*) by Dr. Altemir J. Mossi.

The fresh aerial parts of the plant were air-dried at temperature to 30-35°C for 5 days, after being broken into small pieces. The extract was obtained by water infusion with 20 g of the leaves to 100mL of distilled water for 15minutes at 70°C (20% w/v). After cooling and filtration, the extract was frozen and concentrated by lyophilization.

The antioxidant activity of *Maytenus* spp. was evaluated in vitro by the DPPH method. Briefly, the absorbance of free radical 2.2-diphenyl-1-picryl-hydrazil alone (DPPH control) or in the presence of plant extracts (0.01, 0.025, 0.05, 0.075, 0.1, 0.25 and 0.5 $mg.mL^{-1}$) was measured in 515 nm (Miranda and Fraga, 2006), with ethanol used as solvent and blank. The scavenging activity of extracts upon DPPH radical was calculated as percentage of antioxidant activity (AA%), using the Equation 1:

$$AA\% = 100 - \left\{ \left[\left(\frac{\text{Abs. sample} -}{\text{Abs. white}} \right) \times 100 \right] \div \text{Abs. DPPH control} \right\} \quad (1)$$

The concentration of extract able to scavenge 50% of DPPH radical (IC_{50}) was calculated through regression analysis.

The content of total polyphenolics (TP) present in the extracts was estimated by a colorimetric assay at 691 nm in according with Glasl (1983). The percentages of total phenolics were determined in triplicate. Results were expressed as milligrams of galic acid equivalents by grams of extract (mg $EAG.g^{-1}$) and were calculated using the Equation 2:

$$TP(\%) = \frac{15625 \times Abs}{1000 \text{ x } M} \quad (2)$$

where TP = total polyphenolics (%); Abs = absorbance of each sample; M = mass (g) of extracts.

The correlation between phenolic content and antioxidant activity in three *Maytenus* species was performed in Excel.

This study was conducted in accordance with Federal Law N° 11,794 and was approved by the Research Ethics Committee of URI University (RS, Brazil) upon register numbers 077/TCA/09. Male Wistar rats (*Rattus norvegicus* Bakenhouff) two months old, weighing 120 ± 5 g, were obtained from Laboratory of Animal Experiments of URI University (RS, Brazil). The animals were kept in photoperiod of 12 h of light and dark, minimal noise (< 20 db), ambient temperature (22 ± 2 °C) and had free access to food and water.

For experimental procedures, the animals were distributed randomly in groups of 6 rats each, and received orally treatments of 75 $mg.kg^{-1}.day^{-1}$ of NaCl (Salt solution), 330 $mg.kg^{-1}.day^{-1}$ of vitamin C (Vitamin C), 830 $mg.kg^{-1}.day^{-1}$ extract of each species (Extract) and 830 $mg.kg^{-1}.day^{-1}$ of each extract plus 330 $mg.kg^{-1}.day^{-1}$ of vitamin C (Extract plus vitamin C) for 14 days.

After 14 days the rats were sacrificed in CO_2 chamber. Total blood was collected by cardiac puncture and transferred to tubes containing heparin. An aliquot was centrifuged to separate the plasma (5,000rpm, 15min). The samples of blood and plasma were frozen at-20°C and later used to perform, respectively, the comet assay and carbonyl proteins dosage.

The genotoxicity of *Maytenus* extracts was evaluated by the alkaline comet assay as described by Singh et al. (1988), with minor modifications. A 5mL aliquot of previously collected blood were mixed with 100 µL of 0.75% low-melting point agarose, and immediately spread onto a glass microscope slide pre-coated with 1% normal melting point agarose. Agarose was allowed to set at 4°C for 5min. To remove cell proteins, slides were incubated with ice-cold lyses solution (2.5M NaCl, 10mM Tris, 100mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10.0) at 4°C for at least 1h. To promote DNA unwinding and the exposure of alkali-labile sites, slides were placed on a horizontal electrophoresis unit, covered with fresh buffer (300mM NaOH, 1mM EDTA, pH 13.0) for 20min at 4°C. Electrophoresis was performed in alkaline conditions (pH ~ 13) for 15min at 25V and 300mA. After electrophoresis, slides were neutralized (0.4M Tris, pH 7.5), washed in bi-distilled water, stained with silver solution (0.1% ammonium nitrate; 0.1% de silver nitrate; 0.25% tungstosilicic acid; 0.15% formaldehyde), dried overnight and analyzed by optical microscopy. In order to prevent additional DNA damage, the slides preparation was made in a dark room.

For each animal were prepared two independently slides, being evaluated 100 random cells in each one. Cells were visually scored according to tail length into five classes, from 0 (undamaged, without a tail) to 4 (maximum damage, comets with no heads). A damage index (DI) was assigned to each comet according to its class. The damage index is based on the length of migration and on the amount of DNA in the tail, and it is considered a sensitive DNA measurement. Damage index, can range from 0 (completely undamaged 100 cells×0) to 400 (with maximum damage 100 cells×4).

Evaluation of oxidative damage in plasmatic proteins was based on detection of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (DNPH). Previously collected plasma samples were mixed with acetic glacial acid (0.2mL), incubated on the ice (5min), centrifuged (10,000rpm, 5min) and the supernatant was discarded. The remained precipitate received 1mL of DNPH (10mM) and was incubated at 37°C for 60min. A blank was made using HCl (2M) instead DNPH. Following the samples was mixed in vortex (30s), centrifuged (10,000rpm, 5min) and precipitated proteins dissolved in 6M guanidine. The latter procedure was repeated three times; in the last, the supernatant phase was collected and subjected to carbonyl content determination by measure of absorbance at 370nm (Levine et al., 1990) and total protein quantification by Bradford method (Bradford, 1976).

The results were analyzed statistically using one-way ANOVA plus Tukey´s post-test, with p values < 0.05 accepted as significant.

3. Results and Discussion

In these work the in vitro antioxidant activity and the in vivo genotoxicity of *Maytenus dasyclada* was compared to the reference species *M. aquifolium* and *M. ilicifolia*. As elucidated by DPPH method, *M. dasyclada* as well the two other *Maytenus* spp. investigated, showed in vitro antioxidant activity (Table 1), but *M. ilicifolia* was clearly more efficient as radical scavenger since its IC_{50} is dozens of times smaller. The DPPH result obtained to *M. ilicifolia* is similar to previously published data (Pessuto et al., 2009). In these sense, is possible conclude that *M. dasyclada* have antioxidant capacity comparable to the *M.aquifolium* which, in its turn, acts as scavenger of several oxidants, including O_2^{-}, NO and HCOl (Vellosa et al., 2007).

Total phenols content was also similar in *M. dasyclada* and *M. aquifolium*, both species showing values that are approximately half of *M. ilicifolia* (Table 1). Using another methodology, Pereira et al. (2005) has demonstrated that the yields of phenolic compounds in *M. ilicifolia* can indeed be until three fold higher than in *M. aquifolium* (14 to 20% and 4.23 to 7.53%, respectively). No previous data were found in the literature about total polyphenols content in *M. dasyclada*.

Is interesting to note that in the three species, DPPH IC_{50} is inversely correlated with phenolic results and this correlation shown a good linear regression (Pearson correlation of –0.932) (Figure 1), suggesting the importance of phenolic compounds to antioxidant proprieties of the genus. A similar correlation had previously been demonstrated to ethanolic and aqueous extracts of *M. ilicifolia* using DPPH and phosphomolybdenum assay; being that the antioxidant potential of ethanolic extract was better than aqueous extract and was also proportionally increased by the presence of hydroxylated phenolic groups in the fraction (Pessuto et al., 2009).

Table 1. Antioxidant activity and phenolic content of *Maytenus* spp. aqueous extracts.

Specie	DPPH IC_{50} ($mg.mL^{-1}$)	Phenolic content ($mg\ EAG.g^{-1}$)
M. dasyclada	0.990 ± 0.033	22 ± 1.03
M. aquifolium	0.584 ± 0.021	24 ± 0.96
M. ilicifolia	0.030 ± 0.001	50 ± 1.42

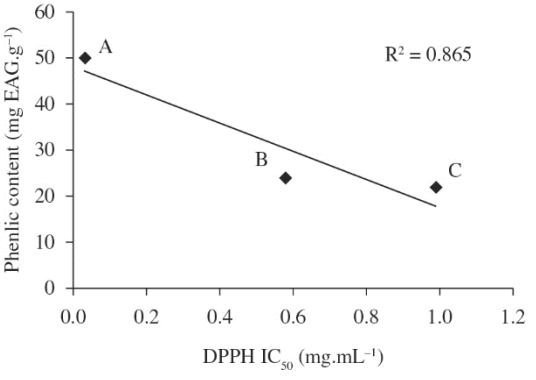

Figure 1. Correlation between phenolic content and antioxidant activity in three *Maytenus* spp. (A) *M. ilicifolia*, (B) *M. aquifolium* and (C) *M. dasyclada*.

Antioxidant-rich fractions (e.g. polyphenols) from plant extracts have been used in experimental models to prove their inhibitory effect on induced ulceration (Vilegas et al., 1999). The protective effect of rutin, a widespread flavonol glycoside, against ethanol induced gastric lesions as well as the inhibition of gastric H^+, K^+ – ATPase by catechins have already been assessed (La Casa et al., 2000). The presence of polyphenols and the antioxidant activity on *Maytenus* spp. can contribute for the efficiency of its infusion as an antiulcer agent and legitimate its medicinal use (Corsino et al., 2003). Besides the best characterized *M. ilicifolia*, other species of the same genus can became, with greater or lesser intensity, pharmacological alternatives.

To ensure their secure use, is important know the biological effects of the plants *in vivo*. In this work the genotoxicity of the *Maytenus* extracts and their ability to oxidize proteins was investigated by the comet assay and carbonyl proteins measure, respectively. The alkaline comet assay is able to detect DNA strand breaks produced by ROS or other toxic substances; it is a sensible and low cost test widely used in the analysis of genotoxicity (Collins et al., 2008). Considering that the maximum DNA damage index (DI) that can be achieved in the comet assay is 400, the results showed that the three *Maytenus* spp. have induced a low and similar level of genotoxicity, which was partially recovered by a co-treatment with vitamin C (Figure 2). In relation to the carbonyl content, *M. dasyclada* and *M. aquifolium* extracts have also a similar effect, promoting the in vivo protein oxidation (Figure 3). Again, this effect could be reverted by combination with vitamin C.

Although the extracts studied had in vitro antioxidant activity, the reduction of DNA damages by vitamin C is an indicative that their slight genotoxic effect in vivo can be mediated by an oxidative mechanism. This observation is in agreement with the pro-oxidant action upon proteins (Figures 2 and 3). *M. ilicifolia* does not cause significant change upon carbonyl content, which can be related with its high content of phenolic compounds comparative to the two other analyzed species (Table 1). The in vivo antioxidant properties of *M. ilicifolia* were previously demonstrated (Melo et al., 2001) and seems be important to the protection of gastric lesions in rats (Baggio et al., 2007).

Despite the use of *Maytenus ilicifolia* as medicinal plant, there are few works that have investigated their genotoxic effects. Camparoto et al. (2002) showed that infusions of *M. ilicifolia* no produce alterations on the cell cycle and chromosomes structure of onion root and bone-marrow cells, while Vargas et al. (1991) demonstrated that the plant is not mutagenic in the AMES test using *Salmonella typhimurium* as biological model. The clinical toxicology of the *M. ilicifolia* has also been investigated in humans and the plant does not altered biochemical e hematologic parameters in the subjects (Tabach et al., 2002).

Particularly, the comet assay was not previously employed with all *Maytenus* spp. target of the present study *in vivo*, only *in vitro* experiments (Schwanzet al., 2013). Although the three species have presented a slight increase

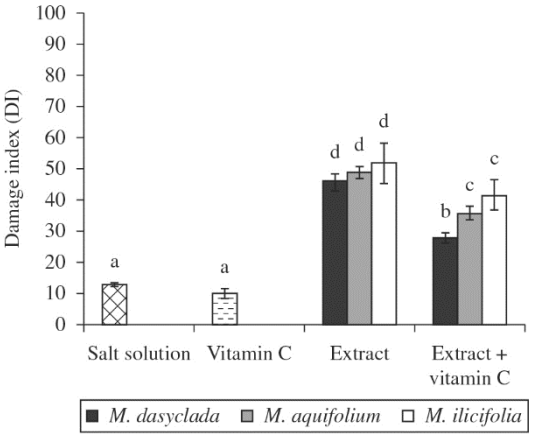

Figure 2. Genotoxicity of the three *Maytenus* species in rats leukocytes. Animals were treated with salt solution (75mg.kg^{-1}.day^{-1}), vitamin C (330mg.kg^{-1}.day^{-1}), extract (830mg.kg^{-1}.day^{-1}) or extract (830mg.kg^{-1}.day^{-1}) plus vitamin C (330 mg.kg^{-1}.day^{-1}) for 14 days. Statistically significant differences between the treatments are indicated by different letters using one-way ANOVA plus Tukey's post test.

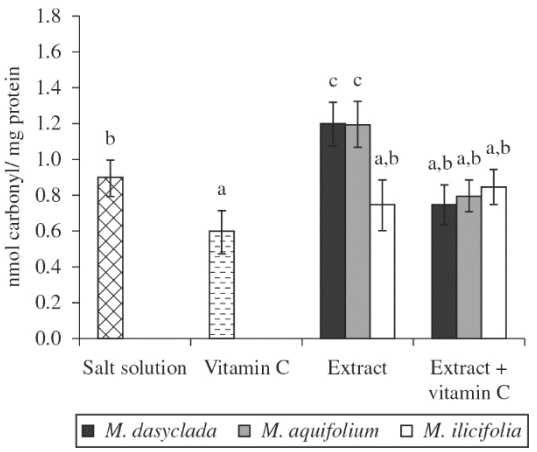

Figure 3. Oxidative damage produced by three *Maytenus* species in plasmatic proteins of rats. Animals were treated with salt solution (75 mg.kg^{-1}.day^{-1}), vitamin C (330 mg.kg^{-1}.day^{-1}), extract (830 mg.kg^{-1}.day^{-1}) or extract (830 mg.kg^{-1}.day^{-1}) plus vitamin C (330 mg.kg^{-1}.day^{-1}) for 14 days. Statistically significant differences between the treatments are indicated by different letters using one-way ANOVA plus Tukey's post test.

in DI, the obtained values are comparable to that seemed in other medicinal plants. Treatment of rats with *Calendula officinalis* during the same time employed in this study results in similar comet levels (DI 34-72 for *C. officinalis* versus 46-52 for *Maytenus* spp.); and even so *C. officinalis* have an antigenotoxic effect that repair the DNA damage caused by methyl methanesulfonate (Leffa et al., 2012). Extracts of *Baccharis trimera*, showing DI value in the same range, have also protective action against DNA

breaks induced by hydrogen peroxide (Rodrigues et al., 2009). Schwanz et al. (2013) evaluating the *in vitro* antioxidative effects of different extracts of *Maytenus dasyclada*, observed reduction in the DI compared to the control, with 100 and10μg/mL, regard less of the fraction of the extract (ethyl-acetate or ethanolic).

4. Conclusions

The aforementioned data permit to conclude that therapeutic application of *M. ilicifolia* is secure in male Wistar rats. However, some cautions relative to dosage and/or time of use as well take into account some particularities of the subjects are necessary. In this work we demonstrated that *M. dasyclada* as antioxidant activity in vitro, and that its genotoxic and pro-oxidant effects in vivo are comparable to the *M. ilicifolia* and *M. aquifolium*.

Acknowledgements

The authors thanks to CNPq, CAPES, FAPERGS and SCIT-RS to financial support.

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