Full Length Research Paper

Trichomonicidal activity of *Maytenus imbricata* (Celastraceae)

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Received 16 January, 2014; Accepted 29 April, 2014

Trichomoniasis, most common non-viral sexually transmitted infection worldwide, is produced by protozoan *Trichomonas vaginalis*. The therapy of choice is metronidazole (MTZ). The drug has undesirable side effects, which may result in treatment discontinuation, leading to further spread of infection and emergence of resistant strains. This feature highlights the importance of studying new trichomonicidal substances. In this context, the importance of plants in relation to the research of new drugs is undeniable. The genus *Maytenus*, distributed throughout Brazil, is the largest of family Celastraceae, including about 80 recognized species with different biological activities. Therefore, the trichomonicidal activity of MTZ and extracts obtained from *Maytenus robusta* leaves and *Maytenus imbricata* roots on the JT strain of *T. vaginalis*, sensitive (JT) and resistant (JTR) to MTZ was investigated. Sample of *T. vaginalis* trophozoites were associated with extracts in 6 increasing concentrations ranging from 0.43 to 13.76 μg/ml. The solid that precipitated from the hexane/ethyl ether - 1:1 extract (SEH), obtained from *M. imbricata* roots proved to be active. This extract also impacted the viability of trophozoites of both strains, with IC₅₀ value surprisingly low (1.09 μg/ml for JT and 1.57 μg/ml for JTR) signaling towards a promising candidate for phytotherapy or for isolation of substance with trichomonicidal activity.

Key words: *Maytenus* spp, metronidazole, *Trichomonas vaginalis*.

INTRODUCTION

Trichomoniasis, which etiologic agent is *Trichomonas vaginalis*, is the most common non-viral sexually transmitted infection worldwide (Johnston and Mabey, 2008). The parasite can be found throughout human genitourinary tract. Transmission occurs primarily through sexual intercourse (Bravo et al., 2010). Most infected men
are asymptomatic, but infection in women has been associated with problems in pregnancy, pelvic inflammatory disease and infertility (Maciel et al., 2004; Carli and Tasca, 2011). Importantly, there is a strong association of trichomoniasis with increased risk of acquisition and transmission of human immunodeficiency virus (Van der Pol et al., 2008). Despite trichomoniasis being a public health problem occurring worldwide, little attention has been given to its control (Burgess and Schwebke, 2004).

The choice treatment is metronidazole, a 5-nitroimidazole used to treat infections caused by protozoa and gram-negative anaerobic bacteria. The drug has some adverse side effects which may lead to poor compliance and treatment discontinuation (Cudmore et al., 2004). In addition, resistant cases of *G. lamblia* and *T. vaginalis* to metronidazole have been well documented over the past century (Uproorf and Uprocroft, 1993; Voolmann and Boreham, 1993). Thus, the research of new substances with trichomonicidal activity aiming not only to overcome resistance but the side effects is a primary objective in the prevention of the disease (Miller and Clardy, 2009).

The importance of plants concerning the identification of bioactive substances is unquestionable (Li and Vederas, 2009). Studies with extracts or isolated compounds are frequent and can be used as herbal medicines or lead to models for the synthesis of more potent and selective similar substances. Recent studies showed isolation of constituents with antitrichomonal, amoebicidal and giardicidal activity from different species of *Maytenus* (Fahmy et al., 2014; Moo-Puc et al., 2014) and the literature reports the plant extracts and isolated secondary metabolites which can inhibit protozoan parasites, such as *Plasmodium*, *Trypanosoma*, *Leishmania*, *Trichomonas* and intestinal worms (Wink, 2012).

In this context, species of the family Celastraceae, mainly of genus *Maytenus*, stand out due to their various biological applications such as anti-inflammatory (Jorge et al., 2004), antibacterial (Lindsey et al., 2006; Santos et al., 2011), antiplasmodial, leishmanicidal (Alvarenga et al., 2008) and possible cytotoxic and antitumor activity (Morita et al., 2008). They are also used in folk medicine as antiseptic, anti-asthmatic, anti-tumor (Jeller et al., 2004; Nakagawa et al., 2004; Perestelo et al., 2010), antiviral (Hussein et al., 1999), in treatment of gastric problems (Baggio et al., 2007; Cipriani et al., 2009) and anti-inflammatory (Sosa et al., 2007).

*Maytenus robusta* has adapted very well to the conditions of the South and Central regions of Brazil and is commonly known as “espinheira santa” or “cancerosa”. This species is used in popular medicine to treat stomach ulcers (Niero et al., 2011). The antulcerogenic, antinociceptive and acetylcholinesterase inhibitory activities of *M. robusta* were previously investigated (Niero et al., 2006; De Andrade et al., 2008; Sousa et al., 2012) but the trichomonicidal activity of extracts from the species of *Maytenus* has not been tested up to the present moment.

The main aim of this study is to further investigate the extracts activity of *M. robusta* and *M. imbricata* about *T. vaginalis* JT strains, sensitive and resistant to MTZ as an alternative therapy to trichomoniasis.

**MATERIALS AND METHODS**

**Parasites and culture conditions**

*T. vaginalis* trophozoites of JT strains were obtained from a symptomatic female patient. Metronidazole resistant JTR derived from JT was maintained in 8 µM metronidazole. Trophozoites were axenically grown in YI-S-32 medium (Diamond et al., 1995) at 37°C with thrice-weekly sub-cultures, assuring their use in the log phase of growth. To determine the best inoculum for association with drugs for 48 h, amounts of trophozoites ranging from $4 \times 10^4$ to $8 \times 10^6$ were evaluated.

**Collection and identification of plant material**

*M. robusta* branches were collected in 2010 in the Itacolomi State Park, Ouro Preto, Minas Gerais, Brazil. A voucher specimen (Exsicata number OUPR 25559) was deposited in the Herbarium Prof. José Badini of the Universidade Federal de Ouro Preto. *M. imbricata* roots were collected at “Morro Santana”, Ouro Preto, Minas Gerais. A voucher specimen (Exsicata number 27780) was deposited in the Herbarium at the Federal University of Viçosa.

**Obtaining solids and crude extracts**

After grinding in a hammer mill, 864.4 g of *M. robusta* leaves and 1.5 kg of *M. imbricata* roots were obtained. *M. robusta* extracts were obtained by cold extraction in exhaustive extractions with hexane (HE), chloroform (CHCl₃), ethyl acetate (EtOAc) and methanol (MeOH). During the evaporation of the solvents in a rotary evaporator there occurred precipitation of solids, which were filtered from Hex (SEH, 4.51 g) and CHCl₃ (SCE, 1.78 g) extracts. After the complete solvent removal, Hex extract (HE, 31.43 g), CHCl₃ extract (CE, 18.49 g), EtOAc extract (EE, 5.74 g) and MeOH extract (ME, 130.27 g) were obtained. *M. imbricata* roots were submitted to exhaustive extraction in Soxhlet apparatus (heating) with solvents in increasing order of polarity: Hex-Et₂O (1:1), AcOEt and MeOH. After filtration and solvent removal by distillation under reduced pressure, the respective solids and extracts were obtained: SEH (solid that precipitated from the hexane/ethyl ether - 1:1 extract), FSEH (hexane/ethyl ether - 1:1 extract), SEAT (solid that precipitated from the ethyl acetate extract), FSEAT (ethyl acetate extract) and SEM (methanol extract). The amounts obtained from plant extracts and solids used in the screening, ranged from 5 to 12 mg.

**Drug susceptibility assays**

A screening-type preliminary test of all crude extracts was initially performed in order to select those with activity against both strains, sensitive (JT) and resistant (JTR). The crude extract (16.5 mg) was dissolved into 1.0 ml dimethylsulfoxide (DMSO) and 100.0 ml aliquots of this solution were diluted in 5 ml culture medium, resulting in stock solution at concentration of 0.33 µg/ml. These solutions were filtered through nitrocellulose membrane 0.22 µm
and added to glass tubes containing trophozoites to achieve final concentration of 34 µg/ml. After 48 h of incubation at 37°C, each tube was evaluated using an inverted microscope (Nikon TS100F). Each assay was performed in triplicate and repeated at least twice using negative control (only trophozoites) and DMSO control (0.2%). Extracts showing activity were later associated to the parasite in increasing concentrations to determine the IC_{50}.

**Determination of IC_{50}**

*T. vaginalis* trophozoites were distributed in glass tubes (Pyrex® 13 x 100 mm) containing culture medium to a final volume of 6 ml. The extract previously active in the screening was added to the culture to obtain test concentrations ranging from 0.43 to 13.69 µg/ml. Trophozoites associated with drugs were grown at 37°C for 48 h. After incubation, the viability was qualitatively verified by observing the mobility and adhesion of trophozoites using inverted microscope and quantitatively for the determination of IC_{50} of JT and JTR strains. MTZ was dissolved in DMSO and an aliquot was added to 5 ml of culture. After filtration in sterilizing nitrocellulose membrane (0.22 µm), aliquots of all solutions were added separately in sterile glass tubes containing trophozoites to test in increasing concentrations ranging from 0.1 to 3.2 µM. These tubes were incubated at 37°C in intervals of 48 h to determine the IC_{50}. All tubes were quantified in hemocytometer. The experiments were performed in triplicate and repeated twice using negative control and DMSO control (0.2 %).

**Statistical analysis**

Simple linear regression analysis (Werkema and Aguiar, 1996) was used to estimate the relationship between the concentration and the percentage of inhibition. The dependent variable was the percentage of inhibition and the independent variable was the concentration. Data on the inhibition percentage for each concentration of test substance were compared by analysis of variance (ANOVA). For validation and subsequent use of the proposed regression equation was held residue analysis and outliers. The adjustment of the model was evaluated by the determination coefficient. From the relationship obtained, the IC_{50} was estimated beyond the confidence interval using the method of inverse regression. To determine the error rate the confidence intervals was used and estimated for the IC_{50} which was obtained using the technique of reverse regression, given a confidence level of 95 and 90%. The Minitab 15 was the statistical software used.

**RESULTS**

The trophozoites inoculum used was 6.0x10^4/ml, since after 48 h of incubation at 37°C, the culture showed formation of monolayer of elongated trophozoites, good vitality and no precipitation (data not shown). In order to investigate for new active drugs against *T. vaginalis* resistant to MTZ treatment, we attempted to achieve in vitro resistant trophozoites to this drug for use during the tests with the extracts. The strain of *T. vaginalis* JT was maintained in axenic culture in the presence of increasing concentrations of metronidazole that started with 1 µM, reaching a final concentration of 8 µM. In the initial screening, MTZ and all extracts were evaluated as for the in vitro trichomonidal activity. None of the extracts obtained from *M. robusta* leaves showed inhibitory effect up to concentration of 34 µg/ml. Among the extracts obtained from *M. imbricata* roots, only SEH proved to be active. Therefore, new studies on the activity in this extract were carried out in order to determine its IC_{50} after an incubation period of 48 h for both JT and JTR strains. The IC_{50} value for SEH on the JT strain was 1.09 µg/ml, ranging from 0.59 to 2.1 µg/ml (confidence interval of 95%). The IC_{50} value for SEH on the JTR was 1.57 µg/ml, ranging from 0.93 to 2.65 µg/ml (confidence interval of 95%). Thus, it was observed that this extract also impacted the viability of trophozoites of JT strain showing high trichomonidal activity, including MTZ resistant strain, JTR (Figure 1).

**DISCUSSION**

Reports of resistance to metronidazole, limited options of drugs for treatment and the occurrence of adverse effects strengthen the need for the development of new trichomonidal drugs. In this context, natural products obtained from plants are a promising area for the discovery of innovative bioactive substances (Miller and Claridy, 2009). Species of the Celastraceae family stand out due to their various biological applications. The study of extracts and compounds isolated from leaves, roots and branches of some species of *Maytenus* presented antioxidant activity. *M. rigidia* stands out in treating infections and inflammations, *M. truncata* showed analgesic and antulcerogenic activity and *M. senegalensis* antiplasmodial activity (Fonseca et al., 2007; Estevam et al., 2009; Malebo et al., 2009).

A pentacyclic triterpene, 3,4-seco-friedelan-3-olic acid, isolated from leaves of *M. imbricata*, has inhibitory activity in the synthesis of adenosine triphosphate (ATP), which may be used in developing natural herbicides (Silva et al., 2007). The extracts (hexane/ethyl ether - 1:1, ethyl acetate and methanol) of *M. imbricata* roots and the isolated triterpenes 11α-hydroxylup-20(29)-en-3-one, tingenone and 6-oxo-tingenol, showed antimicrobial properties were submitted to in vitro assays concerning the bacteria *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and the fungus *Candida albicans* (Rodrigues et al., 2012). It was also observed that tingenone, compound isolated from the roots of *M. imbricata*, present antinociceptive activity when tested on mice (Veloso et al., 2014). Therefore, the trichomonidal activity of extracts and solids obtained from *M. imbricata* roots and *M. robusta* leaves was herein investigated.

Initially, the best inoculum to obtain cultures at exponential growth phase after 48 h of incubation was evaluated. This phase is crucial for obtaining replicas of different concentrations of the test substances associated
with trophozoites. The inoculum of $6 \times 10^4$ trophozoites/ml was used because it showed the formation of monolayer of elongated trophozoites with good vitality and lack of precipitation, considered ideal for identifying the action of extracts on strains. In the screening performed with JT strain sensitive and resistant to metronidazole, only the solid of hexane/ethyl ether - 1:1 extract from *M. imbricata* roots (SEH) showed inhibitory effect. So, other studies were carried out to determine its IC$_{50}$ value against sensitive and resistant *T. vaginalis* strains. The values found were evaluated by descriptive analysis techniques and scatter plots, which showed the trend line of the relationship between extract concentration and the growth inhibition percentage of *T. vaginalis* under study. These results were supported by the simple linear regression analysis method. There were no outliers and the residuals were independent and normally distributed, indicating the adequacy of the chosen model.

The results corroborate those of other studies, showing antimicrobial (Rodrigues et al., 2012), antimalarial, giardicidal (Mena-Rejón et al., 2007; Lhinhatrakool et al., 2011), trypanocidal (Godjiman et al., 1985), HIV, antitumoral (Ravelo et al., 2004) and cytotoxicity activities (Rodrigues et al., 2012), among others for genus *Maytenus* (Lindsey et al., 2006; de Andrade et al., 2007; Santos et al., 2011; Niero et al., 2011; de Araújo Júnior et al., 2013). The potential of natural products for the treatment of diseases is evident in traditional medicine. Compared to other protozoa, investigations of natural products with trichomonacidal activity are rarely found in literature (Frasson et al., 2012). The IC$_{50}$ value of the SEH fraction for JT and JTR strains was surprisingly low, signaling a new candidate for trichomonacidal herbal medicines, as well as for isolation of substance with trichomonacidal activity.

REFERENCES

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