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Original Article

Evaluation of the Activity of *Tontelea micrantha* Extracts against Bacteria, *Candida* and *Mayaro virus*

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Abstract

Objectives: This work aimed to evaluate the antibacterial, antifungal, and anti-*Mayaro virus* (MAYV) activity of leaf and branch extracts from *Tontelea micrantha*. **Materials and Methods:** *T. micrantha* extracts were prepared through the partition of the leaf and branch samples in different solvents. Then, the antibacterial and antifungal activity was assessed against bacterial pathogens and *Candida* sp. by the determination of the minimum inhibitory concentration (MIC) by the broth microdilution method. The activity against anti-MAYV was evaluated through the quantification of the extract concentration that promoted the protection of 50% of the cells after the viral infection. **Results:** The extracts of *T. micrantha* were inactive (MIC >500 µg/mL) against Gram-positive, Gram-negative and *Candida* species at the highest concentration tested (500 µg/mL). Anti-MAYV activity was also not detected, with SI <10, ranging from 1.2 to 3.6. **Conclusion:** Although it is used in traditional medicine, Leaf and branch extracts from *T. micrantha* did not present antimicrobial activity, which could be caused by the antagonistic effect of the compounds present in the extract.

Keywords: Antimicrobial activity, branch extracts, leaf extracts

Introduction

The use of antimicrobials represents one of the most successful ways of chemotherapy in the medical clinic. These drugs were responsible for the control of several infectious diseases, which have been considered as the main cause of mortality and morbidity throughout the history of the humanity.[1] However, infectious diseases remain a major challenge to human health. Currently, it is estimated that about 1 billion people are diagnosed with mycoses annually, and more than 1.5 million die from complications of invasive fungal diseases.[2] Furthermore, infectious diarrhea and pneumonia account for 40% of child deaths worldwide, especially in underdeveloped regions.[3]

Over the past 40 years, only two classes of antibiotics effective against Gram-negative bacteria have been approved for clinical use (e.g., oxazolidinones and cyclic lipopeptides) and the development of new antimicrobials in the future seems unpromising.[3] Currently, there is a scarce therapeutic arsenal for the treatment of fungal infections and the development of new antifungal drugs conflicts with the difficulty of finding compounds which are specific for fungal cells.[4] Similar situation happens with the development of new antivirals. Since viruses are obligate intracellular parasites, finding a drug that has an action against the virus but does not interfere with the basal functions of the cells is pointed out as the main challenge.[5] In this regard, considering the 15 largest companies in the pharmaceutical industry, only 5 of their drugs in the clinical or preclinical research phase are anti-infective agents.[3]

The alarming increase in the microorganism's resistance to the currently available antimicrobials, caused mainly by the long-term exposure to subinhibitory concentrations of these

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drugs, has contributed to making this scenario even more adverse. In recent decades, cases of infections caused by resistant strains have frequently been reported, which reduces the efficacy of available drugs and limits the therapeutic arsenal to a few options, many of which involve compounds with high clinical toxicity and high costs, such as polymyxins.[4] In the USA, as an example, about 25,000 patients die annually due to infections caused by Multidrug resistant microorganisms.[1]

Mayaro virus (MAYV) belongs to the genus *Alphavirus* (Togaviridae Family) and is classified as an emerging virus with the potential emergence of an urban cycle in the Americas. It is an arbovirus closely related to the *Chikungunya virus*, and both have similar symptoms, making their infections difficult to diagnose and distinguish. Severe arthralgia caused by this pathogen can last for weeks or months, and the symptoms are treated with analgesics and nonsteroidal anti-inflammatories. However, there are no specific drugs available to treat MAYV infections.[6]

The use of medicinal plants dates from the beginnings of societies and has spread throughout the world in recent years.[7] About 80% of the population of developing countries depends on traditional medicine for treatment of various diseases, such as skin infections, bacterial and fungal infections, and cold.[8] In addition, many commercially available drugs are structurally based on natural compounds, such as salicylic acid (aspirin), quinine (antimalarial), and *Papaver somniferum* morphine.[9] Considering this scenario, the use of extracts and secondary metabolites originated from plants as possible antimicrobials have gained importance, mainly due to their generalized biological activities, which may be enhanced by different components of the extracts.[10]

The Celastraceae family comprises herbs, shrubs, trees, and lianas distributed in the tropical and temperate regions.[11] Numerous substances with biological activity were isolated from the plants of this family, such as triterpenes, with cytotoxic and antimicrobial activity; alkaloids with immunosuppressive effect; and sesquiterpenes, with antitumor activity.[12] *Tontelea micrantha* (MART. EX SCHULT) A. C. SM. (HIPPOCRATEIOIDEAE – CELASTRACEAE) is a representative species of the Celastraceae family. The alcoholic extract of *T. micrantha* bark is traditionally used in Brazil to treat kidney problems, and the seed oil is considered a potent anti-inflammatory.[13] Mercadante-Simões *et al*.[14] identified the presence of tannins, alkaloids, flavonoids, and terpenoids in the bark of *T. micrantha*, highlighting its potential in the bioprospecting of new herbal medicines. In another study, Mercadante-Simões *et al*.,[15] through the pharmacognostic analysis of *T. micrantha* leaves, identified the presence of steroids, triterpenoids, alkaloids, total phenolics, tannins, flavonoids, catechins, and reducing sugars in this part of the plant. Since tannins and flavonoids are also known for their antimicrobial properties, it is important to investigate the biological potential of this species against pathogens of medical interest.

The need for new, effective, and affordable drugs for the treatment of microbial infections is considered a major public health challenge.[13] Thus, this work aimed to evaluate the antibacterial, antifungal, and anti-MAYV activity of crude extracts of *T. micrantha*.

Materials and Methods

Plant material and preparation of the extracts

Plant material was collected in Montes Claros, Minas Gerais – Brazil and identified by the botanist Dr. Maria Olívia Mercadante-Simões. The voucher specimen (Number BHCB 144.623) was deposited at the Herbarium of the Department of Botany of the University of Minas Gerais.

Leaf and branch samples of *T. micrantha* dried at room temperature were fragmented in knives mill. The branch and leaf were submitted to the extraction by successive maceration with pure organic solvents: hexane, chloroform, ethyl acetate, and methanol. The extractive solvents were recovered using a rotary evaporator leading to the respective extracts. At the time of the bioassay, the extracts were diluted in 20% (w/v) dimethyl sulfoxide (DMSO).

Microorganisms and cell lineage

The microorganisms employed in this work originated from the American Type Culture Collection (ATCC), cordially provided by the Fundação Oswaldo Cruz, Brazil. The MAYV was kindly provided by Ph. D. Maurício Lacerda Nogueira (Faculty of Medicine of São José do Rio Preto/Famerp). The antimicrobial activity of each *T. micrantha* extract was determined using nine Gram-negative bacteria (*Enterobacter cloacae* ATCC 23355, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 4352, *Klebsiella oxytoca* ATCC 0182, *Shigella flexneri* ATCC 12022, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enterica* serovar *typhimurium* ATCC 14028, and *Salmonella choleraesius* ATCC 10708), six Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus agalactiae* ATCC 13813, *Enterococcus faecalis* ATCC 19433, *Bacillus subtilis* ATCC 6051, and *Bacillus cereus* ATCC 11778), and four species of *Candida* (*Candida albicans* ATCC 18804, *C. albicans* 10231, *Candida glabrata* ATCC 2001, *Candida krusei* ATCC 34135, and *C. tropicalis* ATCC 28707). The cytotoxicity and the anti- MAYV evaluations were performed using Vero cells (Kidney cells from an African green monkey) ATCC CCL-81, USA.

Antibacterial activity

The antibacterial activity was evaluated by the determination of the minimum inhibitory concentration (MIC) using the broth microdilution method, according to document M07-A9 of the Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012),[16] with minor modifications. In brief, a concentration range (1–500 µg/ml) of the extracts was prepared by serial dilutions in Mueller-Hinton broth (Kasvi, Brazil). For the bacterial inoculum, isolated colonies cultured in

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Mueller-Hinton agar (Kasvi, Brazil) were suspended in sterile saline solution 0.85% (Proquímicos, Brazil), and the turbidity was adjusted in a spectrophotometer (Biochrom, United Kingdom) according to the 0.5 McFarland scale ($OD_{625\,nm}$ = 0.190–0.210). The bacterial suspension (50 µL) was then dissolved in 10 mL of Mueller-Hinton broth to reach 10^6 CFU/mL of optical density. Bacterial inoculum (100 µL) was added to the microplates and incubated for 24 h at 37°C. The MIC was considered the lowest concentration in which no visible bacterial growth could be observed. Amoxicillin and chloramphenicol (1–500 µg/mL) were used as positive controls and DMSO as a negative control. The assays were performed in triplicate.

Antifungal activity

To evaluate the antifungal activity of the plant extracts against *Candida* species, the broth microdilution method, according to CLSI document M27-A3,[17] was used, with minor modifications. Briefly, the extracts were diluted in Sabouraud Dextrose Broth (Acumedia, Brazil) in microplates at concentrations ranging from 1 to 500 µg/mL. Subsequently, the pre-inoculum was prepared from isolated colonies cultured for 48 h in Sabouraud dextrose agar (Acumedia, Brazil) whose turbidity was adjusted according to the 0.5 McFarland scale (10^6 CFU/mL). The pre-inoculum was then dissolved until the resulting cell density was 10^3 CFU/mL. The microplates were incubated for 48 h at 37°C, and the MIC was considered the lowest concentration visually capable of inhibiting the microbial growth. As a positive control, nystatin was used in the concentration range of 1–500 µg/mL, and DMSO as a negative control. The assays were performed in triplicate.

Cytotoxicity assay

The cytotoxic concentration (CC) to 50% of the cells (CC_{50}) was determined using the indirect MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); Sigma-Aldrich, USA) colorimetric method.[18] In 96-well microplates, Vero cells (5×10^4 cells/well) were implanted and maintained in Dulbecco's Modified Eagle's Minimum Medium (DMEM) (Cultilab, Brazil) with 5% of fetal bovine serum (FBS, Cultilab, Brazil). After 24 h, the cells were treated with different concentrations of the plant extracts (1–500 µg/mL) diluted in DMEM (containing 2.5% FBS) and incubated at 37°C for 48 h. Then, the medium of the wells was replaced by 25 µL of MTT solution (2 mg/mL in phosphate buffered saline 1x), and the plates were incubated for 2 h at 37°C in 5% CO_2, allowing the tetrazolium salt to be metabolized by the viable cells. Then, 100 µL of DMSO was added to the microplates. The optical density of the solution in the microplate wells was spectrophotometrically determined at 492 nm. The CC_{50} represents the extract concentration required to reduce cell viability by 50% when compared to the viability of the untreated cells.

Antiviral assay

The highest concentrations of all extracts, toxic to 50% of the cells (CC_{50}) or nontoxic, were tested for antiviral activity against the MAYV (isolated from *Haemagogus* spp captured on the Belém-Brasília highway). For this, Vero cells (5×10^4 cells/well) in 96-well microplates and the viral inoculum were pretreated for 30 min at 37°C with different concentrations of the extracts, ranging from 1 to 250 µg/mL, viral control was also maintained at 37°C for 30 min. This pretreatment was performed to include all of the stages of the viral cycle in which the compound could act, i.e., in adsorption, penetration, viral replication or virucidal effect. Next, the pretreated cells were infected with the pretreated viral inoculum at a multiplicity of infection of 0.1. After 48 h of incubation, cell viability was also determined using the MTT colorimetric method in a condition equivalent to those used in the cytotoxicity assay. The effective concentration (EC_{50}) represents the extract concentration that promoted the protection of 50% of the cells submitted to viral infection. Ribavirin, a nucleoside analog and nonspecific antiviral, was used as positive control.

Statistical analysis

Regression analysis was performed to analyze the results of the antiviral assays. The mean ± standard deviation of triplicate samples was determined using the statistical software GraphPad Prism® v. 7 (GraphPad Software, Inc. La Jolla, California, USA). A one-way ANOVA test was used to compare treatments.

Results

The antimicrobial potential of six extracts from leaf and branch of *T. micrantha* obtained using solvents of different polarities was evaluated by MIC assay. The MIC > 500 µg/mL [Table 1] suggest that they are inactive against the microorganisms employed. According to Mbaveng *et al.*, the antimicrobial activity of potential drugs is considered to be significant if the MIC values are lower than 10 µg/mL; moderate if 10< MIC <100 µg/mL and low if MIC >100 µg/mL.[19] Amoxicillin, a broad spectrum action-type penicillin that interferes with cell wall biosynthesis showed significant activity for all Gram-positive bacteria, except for *B. subtilis* ATCC 6051, which was insensitive to this drug (MIC >500 µg/mL). For Gram-negative bacteria, this penicillin showed significant activity for *E. coli* ATCC 25992 (MIC 1.95 µg/mL), *S. flexneri* ATCC 12022 (MIC 0.24 µg/mL), *S. choleraesius* ATCC 10708 (MIC 0.24 µg/mL), and *S. typhimurium* ATCC 14028 (MIC equal to 0.49 µg/mL). For *A. baumannii* ATCC 19606, amoxicillin presented low activity, inhibiting this pathogen at the concentration of 125 µg/mL.

The positive control used for the beta-lactamases producing strains (*E. cloacae* ATCC 23355, *K. pneumoniae* ATCC 4352, *K. oxytoca* ATCC 0182, and *P. aeruginosa* ATCC 15442) was the antibiotic chloramphenicol. This drug presented moderate activity against *K. oxytoca* ATCC 0182 and *P. aeruginosa* ATCC 15442, with MIC values of 62.5 and 15.6 µg/mL, respectively. This amphenicol showed significant activity against *E. cloacae* ATCC 23355 (0.9 µg/mL), and *K. pneumoniae* ATCC 4352 (3.9 µg/mL).

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Table 1: Minimum inhibitory concentration of leaf and branch extracts of *Tontelea micrantha* against pathogenic bacteria and fungi

Microorganisms	MIC (µg/mL)*								
	EALE	HLE	MLE	EABE	CBE	HBE	AMOXI	CHOL	NIS
Gram-negative bacteria									
E. cloacae ATCC 23355	>500	>500	>500	>500	>500	>500	-	0.9	
K. pneumoniae ATCC 4352	>500	>500	>500	>500	>500	>500	-	3.9	-
K. oxytoca ATCC 0182	>500	>500	>500	>500	>500	>500	-	62.5	-
P. aeruginosa ATCC 15442	>500	>500	>500	>500	>500	>500	-	15.6	-
E. coli ATCC 25992	>500	>500	>500	>500	>500	>500	1.95	-	-
A. baumannii ATCC 19606	>500	>500	>500	>500	>500	>500	125	-	-
S. flexneri ATCC 12022	>500	>500	>500	>500	>500	>500	0.24	-	-
S. enterica typhimurium ATCC 14028	>500	>500	>500	>500	>500	>500	0.49	-	-
S. enterica choleraesius ATCC 10708	>500	>500	>500	>500	>500	>500	0.24	-	-
Gram-positive bacteria									
S. aureus ATCC 29213	>500	>500	>500	>500	>500	>500	0.98	-	-
S. saprophyticus ATCC 15305	>500	>500	>500	>500	>500	>500	1.96	-	-
S. epidermidis ATCC 12228	>500	>500	>500	>500	>500	>500	0.49	-	
S. agalactiae ATCC 13813	>500	>500	>500	>500	>500	>500	0.98	-	-
E. faecalis ATCC 19433	>500	>500	>500	>500	>500	>500	0.98	-	-
B. subtilis ATCC 6051	>500	>500	>500	>500	>500	>500	>500	-	-
B. cereus ATCC 11778	>500	>500	>500	>500	>500	>500	6.25	-	-
Yeast									
C. albicans ATCC 18804	>500	>500	>500	>500	>500	>500	-	-	2
C. albicans ATCC 10231	>500	>500	>500	>500	>500	>500	-	-	4
C. glabrata ATCC 2001	>500	>500	>500	>500	>500	>500	-	-	4
C. krusei ATCC 34135	>500	>500	>500	>500	>500	>500	-	-	8
C. tropicalis ATCC 28707	>500	>500	>500	>500	>500	>500	-	-	2

The values represent the average of three readings. *MIC: Minimum inhibitory concentration (µg/mL), EALE: Ethyl acetate leaf extract, HLE: Hexane leaf extract, MLE: Methanolic leaf extract, EABE: Ethyl acetate branch extract, CBE: Chloroform branch extract, HBE: Hexane branch extract, AMOX: Amoxicillin, CHOL: Chloramphenicol, NIS: Nystatin, *E. cloacae: Enterobacter cloacae, K. pneumoniae: Klebsiella pneumoniae, K. oxytoca: Klebsiella oxytoca, P. aeruginosa: Pseudomonas aeruginosa, E. coli: Escherichia coli, A. baumannii: Acinetobacter baumannii, S. flexneri: Shigella flexneri, S. enterica choleraesius: Salmonella enterica choleraesius, S. aureus: Staphylococcus aureus, S. saprophyticus: Staphylococcus saprophyticus, S. epidermidis: Staphylococcus epidermidis, S. agalactiae: Streptococcus agalactiae, E. faecalis; Enterococcus faecalis, B. subtilis: Bacillus subtilis, B. cereus: Bacillus cereus, C. albicans: Candida albicans, C. glabrata: Candida glabrata, C. krusei: Candida krusei, C. tropicalis: Candida tropicalis, S. enterica typhimurium: Salmonella enterica typhimurium*

The MIC results for *Candida* species are presented in Table 1. Compared with the nystatin control (MIC range 2–8 µg/mL), fungal pathogens did not show sensitivity to the fungistatic effect of leaf and branch *T. micrantha* extracts.

Regarding the antiviral potential of the extracts of *T. micrantha*, the results [Table 2] show that all extracts had some activity in protecting Vero cells against MAYV. However, none of them showed a promising effect against MAYV infection, since the relation between the CC_{50} and the EC_{50} that inhibits viral replication (selectivity index [SI]) was less than 10, which indicates that the extracts presented toxicity.[20] The hexane leaf and branch extracts were the most cytotoxic and less effective against infection, therefore less selective. Ribavirin, the positive control, showed low inhibition against the MAYV.

Discussion

Recently, many studies have shown the antimicrobial activity of several extracts and secondary metabolites of plants conventionally used in the traditional medicine, which contributes to the discovery of new bioactive compounds.[21] However, there are no reports in the literature about the phytochemical and antimicrobial activity of *T. micrantha* to compare and discuss the results obtained in this work.

Chen *et al.*[22] isolated flavonoids from the roots and stems of *Tripterygium wilfordii* (Celastraceae) which were active against *Cryptococcus neoformans*, *P. aeruginosa*, vancomycin-resistant *E. faecalis*, and methicillin-resistant *S. aureus*. Mokoka *et al.*[23] isolated six different triterpenes from the leaves of *Maytenus undata* (Celastraceae) and among them, triterpenes, 3-oxo-11α-hydroxyolean-12-ene-30-oic acid and 3,11-dihydroxyolean-12-ene-30-oic acid showed the best activity (MIC value ranging from 24 to 63 µg/ml) for two Gram-positive species (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212), two Gram-negative species (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853), and clinical isolates of *C. albicans* and *C. neoformans*.

Kloucek *et al.*[24] studied the antimicrobial activity of the ethanol extracts of medicinal plant barks used in Peruvian Amazon in

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Table 2: Citotoxicity and anti-Mayaro activity of extracts of *Tontelea micrantha*

Extracts	CC_{50} (μg/mL)[a]	EC_{50} (μg/mL)[b]	SI[c]
EALE	201.5±33.2	55.8±2.3	3.6
HLE	148.4±12.4	50.1±5.8	2.9
EABE	224.4±37.1	63.7±5.4	3.5
CBE	327.9±43.5	91.0±14.4	3.6
HBE	177.7±12.5	143.7±31.5	1.2
Ribavirin (positive control)	523.1±42.5	118.8±1.9	4.4

[a]50% cytotoxic concentration, [b]50% effective concentration of viral replication, [c]Selectivity Index: ratio between substance's CC_{50} and EC_{50}. EALE: Ethyl acetate leaf extract, HLE: Hexane leaf extract, EABE: Ethyl acetate branch extract, CBE: Chloroform branch extract, HBE: Hexane branch extract, SI: Selectivity index

concentrations ranging from 16 to 0.0156 mg/mL. These authors showed that the plants possessed significant antimicrobial activity. However, of all the plants tested, the broadest spectrum of action was obtained for *Maytenus macrocarpa* (Celastraceae), which showed activity against bacteria and fungi in the concentration range from 125 to 250 μg/mL.

Dhayalan *et al*.[25] evaluated the presence of secondary metabolites and the antimicrobial activity of the ethanol and chloroform extracts of *Spathiphyllum cannifolium* (Araceae). The phytochemical screening showed the presence of steroids, triterpenes, flavonoids, alkaloids, saponins, glycosides, and tannins. The antimicrobial activity revealed that the leaf extracts inhibited *C. albicans*, *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa*. In this study, the chloroform extract showed activity for all the pathogens.

When evaluating the antimicrobial activity by the disk diffusion method of the acetone extract of seven Cuban plants against *S. aureus*, *E. coli* and *C. albicans*, Abreu *et al*.[26] noticed that most plants did not present or presented low antimicrobial activity against the species employed. Such a result reinforces the fact that, although those plants are commonly used in traditional medicine, a bioprospection of the biological activities of such species should be performed to determine their real potential.

Only compounds with SI >10 are considered safe and nontoxic due to the distance between the pharmacological dose value and the toxic dose value. In this direction, none of the extracts of *T. micrantha* showed promising anti-MAYV activity, demonstrating an SI value lower than the ribavirin (a nonspecific antiviral). Spindola *et al*.[27] evaluated the anti-MAYV activity of *Cassia australis* (Fabaceae) extracts and obtained SI values of 20 and 33 to the ethanolic and butanolic extracts, respectively, which indicates a good antiviral effect.

Santos *et al*.[28] evaluated the anti-MAYV activity of the ethanolic and butanolic extracts of *Bauhinia longifolia* (Fabaceae) and the flavonoids quercetin and quercetin 3-O-glycosides isolated from these extracts. The ethanolic extract showed the highest SI value (623), while ribavirin showed an SI of 8. This result indicates that the concentration needed for the extract to have antiviral activity is lower than the concentration that causes the toxic effect on the cells.

In the assay conditions, the leaf and branch extracts of *T. micrantha* did not show antibacterial and antifungal activities. However, there is a mixture of substances in the extracts, and one compound may be playing an antagonistic effect on the other, inhibiting its activity. It is necessary to isolate the constituents of each extract to evaluate their antimicrobial effect, which was not part of this study.

Conclusion

Leaf and branch extracts from *T. micrantha* were assayed against many bacteria and fungi but, under the conditions employed, they did not present activity. Further studies concerning the isolation and antiviral activity of the isolated compounds are currently performed in our laboratory, and the results will be reported in due course.

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Conflicts of interest

There are no conflicts of interest.

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