Antimicrobial activity of triterpene acids against phytopathogens

Atividade antimicrobiana de triterpenos ácidos contra fitopatógenos

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Arthur Alves Ragozoni
Mestre em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: realarthurragozoni@hotmail.com

Maria Anita Lemos Vasconcelos
Graduada em Biomedicina
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: malvasconcelos@yahoo.com.br

Daiane Ferreira Galvão
Graduada em Farmácia
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: day-ferreira@hotmail.com

Nayanne Larissa Cunha
Doutora em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: dranayannecunha@gmail.com

Heloiza Diniz Nicolella
Mestre em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: helo_nicolella@hotmail.com

Ana Helena Januário
Doutora em Química
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: ana.januario@unifran.edu.br

Patricia Mendonça Pauletti
Doutora em Química
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: patricia.pauletti@unifran.edu.br

Márcio Luís Andrade e Silva
Doutor em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: marcio.silva@unifran.edu.br

Denise Crispim Tavares
Doutora em Genética
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: denise.crispim@unifran.edu.br

Carlos Henrique Gomes Martins
Doutor em Biociências e Biotecnologias Aplicadas à Farmácia
Universidade Federal de Uberlândia
Avenida João Naves de Ávila,2121, CEP 38.400-902, Uberlândia, MG
E-mail: carlos.martins2@ufu.br

Wilson Roberto Cunha
Doutor em Química
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: wilson.cunha@unifran.edu.br

ABSTRACT
Phytopathogenic microorganisms, responsible for causing diseases in various types of plantations, have an immense impact on crops, reducing food production, which is one of the main problems of agriculture. Antibiotics in association with cupric fungicides have been commonly used to solve this problem, but result in toxic residues to humans, animals and the environment, in addition to not being as effective as expected. Natural products, such as triterpenes, have become an important source of new substances to fight pathogens. In this study, the triterpenes ursolic acid, oleanolic acid and gypsogenic acid were re-isolated from *Miconia stenostachya*. The identification of all substances was carried out based on data obtained from ^{1}H NMR, ^{13}C-NMR and/or comparison with authentic standards. In the assays of antimicrobial activity against nine phytopathogenic bacteria, the triterpene gypsogenic acid was the most effective with a MIC value of 3.12; 25 and 100 µg/mL, for bacteria *Ralstonia solanacearum*, *Pseudomonas syringae* and *Streptmyces scabiei*, respectively. The cytotoxic activity results of gypsogenic acid in GM7492A cells (human fibroblasts) indicated that the substance promoted toxic effects in the strain only at higher concentrations (above of 500 µg/mL – 1,027.3 µM).

Keywords: triterpenes, ursolic acid, oleanolic acid, gypsogenic acid, antimicrobial activity, phytopathogens.

RESUMO
Os microrganismos fitopatogênicos, responsáveis por causarem doenças em vários tipos de plantações, causam um imenso impacto no aproveitamento das colheitas, reduzindo a produção de alimentos que é um dos principais problemas da agricultura. O que vem sendo comumente utilizado para a resolução deste problema, é a associação de antibióticos a

fungicidas cúpricos, que resultam em resíduos tóxicos ao ser humano, animais e ao meio ambiente, além de não apresentarem os resultados efetivos esperados. Os produtos naturais, como os triterpenos, têm se tornado uma importante fonte de novas substâncias no combate a patógenos. Neste estudo, a partir do vegetal *Miconia stenostachya* foram reisolados os triterpenos ácido ursólico, ácido oleanólico e ácido gipsogênico A identificação de todas as substâncias foi realizada com base nos dados obtidos de RMN-^{1}H, RMN-^{13}C e/ou comparação com padrões autênticos. Nos ensaios da atividade antimicrobiana frente a nove bactérias fitopatogênicas, o triterpeno ácido gipsogênico foi o mais efetivo apresentando CIM de 3,12; 25 e 100 µg/mL, para as bactérias *Ralstonia solanacearum*, *Pseudomonas syringae e Streptmyces scabiei*, respectivamente. Os resultados da avaliação da atividade citotóxica do ácido gipsogênico em células GM7492A (fibroblastos humanos) indicaram que a substância promoveu efeitos tóxicos na linhagem somente em concentrações mais elevadas (acima de 500,00 µg/ml – 1027,3µM).

Palavras-chave: triterpenos, ácido ursólico, ácido oleanólico, ácido gipsogênico, atividade antimicrobiana, fitopatógenos.

1 INTRODUCTION

The diseases caused by phytopathogenic microorganisms are responsible for significant loss in agricultural production, due to its rapid dissemination, which makes its control difficult [1,2,3]. The management of these diseases in plants is a serious problem in agriculture[4,5]; moreover, many of these phytopathogenic microorganisms have acquired resistance to most synthetic bactericides and fungicides[6], as well as the ability to spread over great distances through contaminated or infected seeds[7-9]. In addition, the indiscriminate use of pesticides based on antibiotics in cultivated plants has led to the presence of toxic residues in both man and animals[10,11].

Antibiotics combined with cupric fungicides have usually been recommended for the control of phytopathogenic diseases; however, results are not satisfactory. The use of antibiotics in agriculture has been banned in several countries due to its natural toxicity and its negative impact both on the culture itself and on the environment[12].

It is therefore important to search for new alternative agents to control pathogenic diseases in plants, considering that several researches have reported the use of active ingredients obtained from special plant metabolites[13-15].

In this regard, the possibility of using natural antimicrobial agents is an attractive alternative to control or reduce the bacterial and fungal load in food products, since they are biodegradable, ecological, and biologically safe[5-7].

The aim of the present work was to evaluate the antimicrobial activity of triterpene acids isolated from the plant species *Miconia stenostachya* (Melastomataceae) against nine phytopathogenic bacteria.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL, EXTRACTION AND ISOLATION

M. stenostachya was collected along the Franca-Claraval highway, São Paulo (Brazil) and identified by Dr. Angela Borges Martins, Instituto de Biologia, UNICAMP, Brazil; the specimen (UEC 65132) was deposited in the Herbarium of the same Institute. The aerial parts of the plant were dried at 40°C and the dried material was powdered (1.0 Kg) and extracted by maceration with methylene chloride (5L, 3 days x 3) and ethanol (5L, 3 days x 3) at room temperature. Previous studies carried out in our facilities with these extracts facilitated the isolation of the triterpenes ursolic acid, oleanic acid and gypsogenic acid[16].The structural elucidation of these isolated compounds was performed using spectroscopic methods (MS, ^{1}H and ^{13}C NMR) in comparison with published data[17-19].

2.2 ANTIMICROBIAL ASSAY

All strains used to determine the antibacterial activity of the samples were provided by Prof. Dr. Nilvanira Donizete Tebaldi, researcher at the Institute of Agricultural Sciences, Federal University of Uberlândia (UFU), Prof. Dr. Antônio Carlos Moringoni, from the Department of Plant Production-Phytosanitary Defense, from Universidade Estadual Paulista Júlio de Mesquita Filho (FCA/UNESP-Botucatu/SP) and Prof. Dr. Suzete Aparecida Lana Destéfano, researcher at the Instituto Biológico de Campinas/SP. These phytopathogenic bacteria are presented in Table 1, along with other characteristics and information regardong their origin.

The minimal inhibitory concentration values (MIC; lowest concentration of the compound capable of inhibiting the microorganisms´ growth) was determined in triplicate using the microdilution broth method in 96-well microplates[20,21]

The samples were dissolved in DMSO (dimethyl sulfoxide) at 0.5 mg/mL, followed by dilution in Kado & Heskett medium 523[22], obtaining concentrations ranging from 400 to 1 µg/mL. The final DMSO content was 5% (v/v), and this solution was used as negative control. The inoculum was adjusted for each organism to obtain a cell concentration of $5x10^{5}$ colony forming units (CFU). mL^{-1} according to guidelines of the Clinical Laboratory Standards Institute. One inoculated well was included to control the adequacy of the broth.

In order to ensure medium sterility, one non-inoculated well containing no antimicrobial agent was also included in the assays. The microplates were sealed with a plastic film and incubated at 28°C for 24 h. Following, resazurin (30 µL) in aqueous solution (0.02%) was added to the microplates.

Table 1: Microorgnism strains used in this work.

Microorganism	Code	Gram Strain	Origin
Xanthomonas axonopodis pv. passiflorae	UFUA45	Negative	Passion fruit leaves Tupaciguara-MG
Xanthomonas campestris pv. campestris	247	Negative	Instituto Biológico Campinas-SP
Pantoea ananatis	UFUB13	Negative	Corn leaves Planaltina de Goiás- GO
Burkholderia cepacia	UFUD15	Negative	Onion bulb Juliana-MG
Ralstonia solanacearum	UFUF7	Negative	Tomato stem Patrocínio-MG
Pseudomonas syringae pv. garcae	2212	Negative	Instituto Biológico Campinas-SP
Clavibacter michiganensis subesp. *michiganensis*	1132	Positive	Instituto Biológico Campinas-SP
Streptmyces scabies	2396	Positive	Instituto Biológico Campinas-SP
Curtobacterium flaccumfaciens **pv. flaccumfaciens**	Feij - 3161	Positive	Carioca beans Castro-PR

2.3 EVALUATION OF GYPSOGENIC ACID CITOTOXICITY

Evaluation of the cytotoxic activity was performed in the *in vitro* test system XTT colorimetric assay using the non-tumor human fibroblast strain (GM07492A cells)[24].

The strains were subcultured and an approximate amount of $1x10^4$ cells was seeded in 96-well microplates, each well containing 100 µL of complete culture medium. After 24 hours, the cells were treated with concentrations of gypsogenic acid ranging from 15.6 µg/mL (45.3 µM) to 2,000 µg/mL (5,808.6 µM), in addition to the negative (without treatment), solvent (dimethylsulfoxide, DMSO, Sigma-Aldrich, 0.4%) and positive (DMSO, 25%) controls. The plates were then incubated in an oven at 36.5ºC for 24 hours, the culture medium was removed and the cells washed with 100 µL of PBS to remove the treatments. After washing, 100 µL of Ham F10 culture medium without phenol red (Cultilab) and 25 µL of XTT solution (Roche) were added to each well. The microplates were incubated in an oven for 17 hours. The absorbance of the samples was determined using a multi-plate reader (Asys UVM340, software MikroWin 2000®) at a wavelength of 450 nm and a reference length of 620 nm. The experiments were carried out in triplicate.

3 RESULTS AND DISCUSSION

The chemical structures of the isolated triterpenes ursolic acid (**1**), oleanolic acid (**2**) and gypsogenic acid (**3**) used in the biological assays are presented in Figure 1.

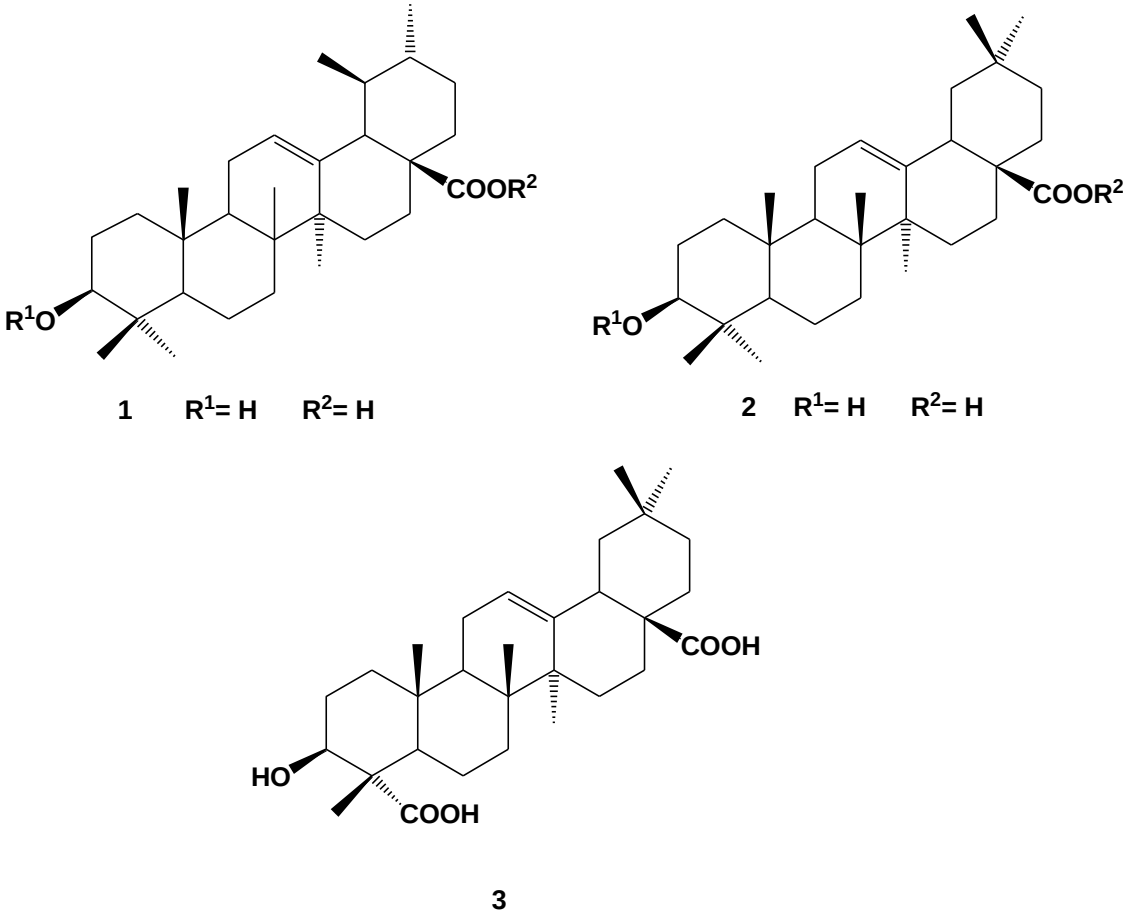
The effect of these three triterpene acids against several phytopathogenic microorganisms was investigated in the present work. The MIC values displayed by these metabolites are shown in Table 2.

The MIC values obtained for the isolated triterpenes indicate ursolic acid as the most effective against *Xanthomonas axonopodis*, *Xanthomonas campestris* and *Clavibacter michiganensis* with a MIC of 50, 100 and 50 µg/mL, respectively. Regarding *Ralstonia solanacearum*, *Pseudomonas syringae* and *Streptmyces scabiei*, the triterpene gypsogenic acid was the most effective, with a MIC of 3.12; 25 and 100 µg/mL, respectively. Regarding the analyzed bacteria and evaluated triterpenes, the best result was obtained for gypsogenic acid against *Ralstonia solanacearum* with a MIC of 3.12 µg/mL. It should be noted that the mixture of oleanolic acid and ursolic acid was not able to potentiate their antimicrobial activity.

Xanthomonas axonopodis pv. Passiflora[25] is a gram-negative phytopathogenic bacterium, the causative agent of bacterial spot disease in passion fruit[26], which is one of the most important in the culture, reducing its period of commercial exploitation[27]. On the other hand, *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson is a gram-negative phytopathogenic bacterium, the causative agent of black rot disease in the Brassicaceae (or Cruciferae) family, including all cultivated species of the Brassica genus[27,28]. In Brazil, the following stand out: Broccoli (*Brassica oleracea* var. Italica); Cauliflower (*Brassica oleracea* var. Botrytis); Leaf kale (*Brassica oleracea* var. Acephala); Cabbage (*Brassica oleracea* var. Capitata), all of relevant socioeconomic importance and essential for health and human nutrition[29]. This phytopathogenic bacterium causes a significant reduction in production and product quality[30].

Ralstonia solanacearum (Smith 1896)[31], is a gram-negative phytopathogenic bacterium that is the causative agent of bacterial wilt, one of the most destructive plant diseases in the world, classified as the second most agronomically important phytopathogenic bacteria[5]. In Brazil, the most susceptible species belong to the Solanaceae family and include potato, tomato, pepper, eggplant, tobacco and jiló, as well as banana, heliconia (caeté or banana from the bush), eucalyptus and castor bean, among others[32,33].

Figure 1. Chemical structures of the tritepenes used in the biological assays



1 R^1= H R^2= H **2** R^1= H R^2= H

3

In a comparative study of three screening techniques[34], it wasconcluded that the broth microdilution test (MIC) was the best option to assess antimicrobial activity. This result corroborates the choice of the test for this work.

Considering that already existing pesticides on the market today are not specific, that is, are fungicides with a bactericidal effect, the use of natural products would be a great advance for the control and management of diseases caused by these phytopathogenic bacteria. And, above all, a specific product would be recommended to the rural producer and, certainly, be less aggressive to the environment.

Thus, the possibility of using natural antimicrobial agents is an attractive alternative to control or reduce the bacterial and fungal load in food products[35,36].

Table 2: *In vitro* antibacterial activity (Minimum Inhibitory Concentration, µg/mL) of the triterpene acids against bacteria phytopathogens

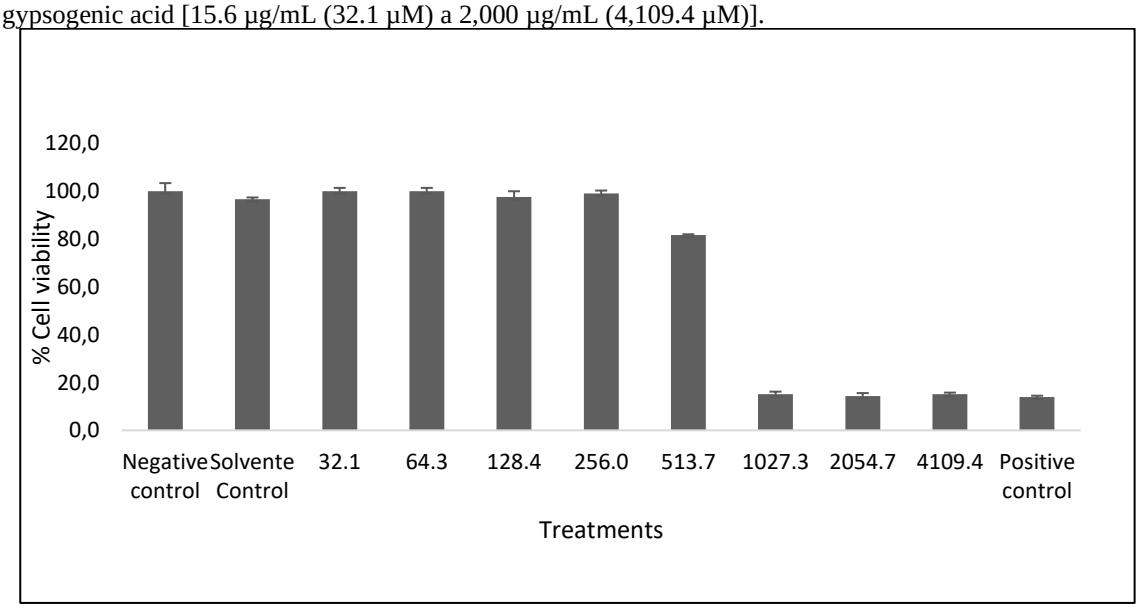
Microorganisms	Gypsogenic acid (3)	Oleanolic acid (2)	Ursolic acid (1)	Mixture (1) + (2)
Xanthomonas axonopodis pv	200	400	50	400
Xanthomonas campestris pv	200	400	100	400
Clavibacter michiganensis	400	>400	50	>400
Pantoea ananatis	100	400	100	400
Burkholderia cepacia	400	400	400	200
Ralstonia solanacearum	3.12	6.25	100	100
Pseudomonas syringae pv.	25	50	50	50
Streptmyces scabies	100	200	400	400
Curtobacterium flaccumfaciens pv. flaccumfaciens	200	200	200	200

**Controls µg/mL: *Echerichia coli* ATCC 25922, tetraciclina 0,7375
Staphylococcus aureus ATCC 29213, tetraciclina 0,3688

The results obtained from the antiproliferative activity assay of gypsogenic acid are shown in Figure 2, in which the curve represents the average of three independent experiments. The cultures were treated in the concentration range of 15.6 to 2,000 µg/mL. The results revealed cytotoxicity absence between the concentrations of 15.6 µg/mL (32.1 µM) and 125 µg/mL (256.8 µM) and were toxic from 500 µg/mL (1,027.3 µM).

The results obtained in the present assay indicate that gypsogenic acid promoted toxic effects in the GM7492A strain (human fibroblasts) in higher concentrations (above 500 µg/mL – 1,027.3 µM).

Figure 2. Viability of GM7492A cells (human fibroblasts) after exposure to different concentrations of gypsogenic acid [15.6 µg/mL (32.1 µM) a 2,000 µg/mL (4,109.4 µM)].



4 CONCLUSION

The possibility of using natural antimicrobial agents is an attractive way to control or reduce the bacterial and fungal load in food products[35,36]. However, information about the specific antimicrobial activity of the main compounds found in plant extracts is still scarce[37]. There are several categories of secondary metabolites with possible antimicrobial action in plant extracts, including terpenes, phenols and nitrogen compounds[38]. Additionally, the possibility of synergism between these molecules can increase the antimicrobial efficiency of several plant types. In view of the obtained results, the search for new products to control phytopathogenic bacteria was evidenced analyzing the contribution of some of the selected substances in the present study.

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