Capter 119

Antimicrobial activity of the natural compounds rosmarinic acid, usnic acid and difrataic acid against phytopathogens

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ABSTRACT
The present study aimed to re-isolate, identify and investigate the anti phytopathogenic potential of a series of secondary metabolites obtained from two diverse botanical sources. From the acetonic extract of *Usnea steineri*, usnic acid and difractaic acid were obtained. From the *Rosmarinus officinalis* extract, rosmarinic acid was re-isolated. The identification of all substances was performed based on data obtained from ^{1}H NMR, ^{13}C NMR and/or comparison with authentic standards. In the tests of the antimicrobial activity against nine phytopathogenic bacteria, the usnic acid was promising, with the best results obtained were against the bacteria *Xanthomonas axonopodis* and *Xanthomonas campestris* with MIC of 3.12 µg / mL and 6.25 µg/mL, respectively. In the survival studies carried out with the usnic acid against *Xanthomonas axonopodis* it was found that in the concentration of 3.12 µg / mL only after 48 hours, the usnic acid was bactericidal for the studied species. The results of the evaluation of cytotoxic activity of usnic acid in GM7492A cells (human fibroblasts) revealed that there were no significant differences between cultures treated with usnic acid (15.6 µg/ml to 2000.0 µg/ml) and the negative control, showing absence cytotoxic effects at the tested concentrations. Because of the results, it can be concluded that usnic acid is a promising compound for the development of a new bactericide for the control of some phytopathogens.

1 INTRODUCTION

Since the origin of agriculture, the creation of pest control methods has been a challenge for man. Plant diseases are caused by pathogens such as fungi, bacteria, nematodes and viruses, with fungi being the parasites that cause the greatest impact on diseases and losses in agricultural production. The most common fungal diseases include powdery mildew, leaf rust, root blight, crown rot, damping off, anthracnose and vascular wilt (MDEE et al., 2009; FONSECA et al., 2015) causing significant losses in agriculture, destruction of grains during storage, decrease in nutritional value and, sometimes, production of mycotoxins harmful to humans and animals (VELLUTI et al., 2004; NAGHETINI, 2006). Many investigations have been conducted to detect new sources of bioinsecticides to control pests in cultivated plants and stored products. However, despite the growing demand for more selective and safe products, few formulations have been developed and made available on the world market (CARVALHO et al., 2015).

The control of these diseases in agriculture has been intensified, being carried out basically through the use of synthetic products. When these products are used rationally, satisfactory results can be obtained, however, their indiscriminate adoption has caused problems of human and environmental contamination and has caused the emergence of pathogens resistant to these chemical products (GHINI & KIMATI, 2000). According to Ribas and Matsumura (2009), pesticides can be defined as natural or synthetic chemical substances, used to kill, control or somehow combat pests, diseases and invasive weeds in crops, constituting an important means of control over agroecosystems (DIAS, et al., 2010).

The search for substitutes for synthetic products finds in plants a very promising alternative of economic and ecological interest (SOUZA et al., 2007), where the use of substances extracted from plants and with possible activity in the inhibition of phytopathogens could represent an option in the disease control in the field.

Brazilian agriculture occupies a prominent position in the supply of products of plant origin due to advances in research and technologies that allow for increased productivity, in addition to the elaboration of more sophisticated agricultural products (PASTRO et al., 2012).

The Chemistry of Natural Products represents, within the area of research with plant species, a point of great importance and value. The diversity of active substances in plant species has motivated the development of research involving the use of plant extracts, essential oils and bioactive substances to explore their fungicidal and bactericidal properties. In the literature, there have been records of the efficiency of plant extracts and isolated substances, obtained from several botanical species, in promoting the inhibition of the development of several phytopathogens (CELOTO et al., 2008; FERREIRA et al., 2012; SALES et al.; 2016; MA et al., 2013).

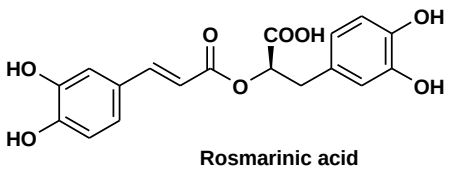
2 MATERIAL AND METHODS

2.1 PLANT MATERIAL, EXTRACTION AND ISOLATION

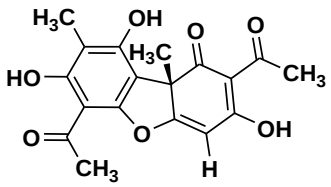
The lichen *Usnea steineri* Zahlbr was collected in the Jataí Reserve-Luís Antônio-SP with authorization provided by COTEC (Scientific Technical Commission) of Instituto Florestal de São Paulo. The aerial parts of *Rosmarinus officinalis* Linn. were collected at the site owned by Prof. Dr. Wagner A. Bernardes, located in the urban perimeter of the Patrocínio city, in the western portion of the state of Minas Gerais, in the physiographic zone of the Triângulo Mineiro and Alto Paranaíba. The identification of the plants was carried out by Prof. Dr. Milton Groppo from the Department of Botany at FFCLRP-USP and the exsiccates were deposited in the Department's Herbarium.

The collected material (*U. steineri*) was dried and stabilized in a circulating air oven (40°C) and ground in a knife mill to a powder form (300 g), which was subsequently subjected to extraction by maceration with the solvent acetone (3 liters, room temperature). The extraction process was repeated three times with an interval of one week between them. All the material resulting from the maceration process was filtered and concentrated under reduced pressure at a temperature of 40ºC, using a rotary evaporator until the complete elimination of the solvent. The dry crude extract (18.44 g) was placed in an amber bottle with a lid and kept in a refrigerator. The phytochemical studies carried out with this extract allowed the isolation of the compounds usnic acid and difractaic acid (Figure 1). To obtain rosmarinic acid (Figure 1), 200 g of powder from the leaves of the plant *R. officinalis* were used. This was subjected to an extraction process by maceration (room temperature) for seven days using water/acetic acid (85:15 v/v). The maceration product obtained was filtered and its pH adjusted to 10 by adding a calcium hydroxide solution. A precipitate (27g) was formed which was purified on a Sephadex LH-20 column, resulting in 7.5g of solid material of yellow color coded as RA.The structural elucidation of these isolated compounds was performed using spectroscopic methods (MS, ^{1}H and ^{13}C NMR) in comparison with published data (KÖNIG & WRIGHT,1999; KHUNT et al.,1994; HONDA,1997).

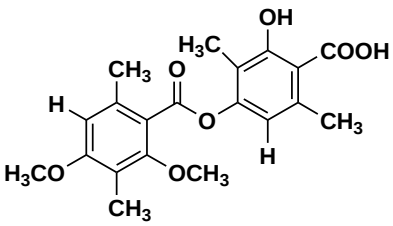
Figure 1. Chemical structures of the isolated compounds



Rosmarinic acid



Usnic acid



Difractaic acid

2.2 ANTIMICROBIAL ASSAY

2.2.1 Strains of phytopathogenic bacteria used in the study

To determine the antibacterial activity of the samples, strains provided by Prof. Dr. Nilvanira Donizete Tebaldi, a researcher at the Institute of Agricultural Sciences, at the Federal University of Uberlândia (UFU), Prof. Dr. Antônio Carlos Moringoni, from the Department of Plant Production - Phytosanitary Defense, from the São Paulo State University Júlio de Mesquita Filho (UNESP-Botucatu/SP) and Profa. Dr. Suzete Aparecida Lana Destéfano, researcher at the Biological Institute of Campinas/SP. These phytopathogenic bacteria are shown in Table 2, along with other characteristics and information about their origin. Other bacteria from our Microbiology Laboratory (LAPEMA) were also used.

Table 2: Strains of phytopathogenic bacteria used in the experiment

Strains	Laboratory code	Gram stain	Origin
Xanthomonas axonopodis pv. passiflorae	UFUA45	Negative	Passion fruit leaves in the city of Tupaciguara MG (2009)
Xanthomonas campestris pv. campestris	247	Negative	Biological Institute of Campinas/SP
Pantoea ananatis	UFUB13	Negative	Corn leaves in the city of Planaltina de Goiás GO (2010)
Burkholderia cepacia	UFUD15	Negative	Onion bulb in the city of Santa Juliana MG (2012)
Ralstonia solanacearum	UFUF7	Negative	Tomato stalk in the city of Patrocínio MG (2014)
Pseudomonas syringae pv. garcae	2212	Negative	Biological Institute of Campinas/SP
Clavibacter michiganensis subesp. michiganensis	1132	Positive	Biological Institute of Campinas/SP
Streptmyces scabies	2396	Positive	Biological Institute of Campinas/SP
Curtobacterium flaccumfaciens pv. *flaccumfaciens*	Feij - 3161	Positive	Isolate of Carioca Beans from the city of Castro-PR

2.2.2 Determination of the minimum inhibitory concentration (MIC) in assays with phytopathogenic bacteria

A sterilized inoculation loop was used to prepare the inoculum. 24 to 72 hour cultures of phytopathogens, indicators (developed in Kado and Heskett's Medium 523 prepared in the laboratory) were transferred to tubes containing 0.9% saline solution. The suspensions were standardized in a spectrophotometer and 1 mL was transferred to another tube containing 9 mL of saline. Then, 2 mL of this inoculum was added to a tube containing 10 mL to provide a concentration of 5 x 105 CFU.mL^{-1}, as provided for in CLSI, 2009. The 0.5 Mc Farland scale was also used in the inoculum standardization process.

To standardize the density and obtain the inoculum, the strains stored in a freezer at minus 10 ºC were resuspended in liquid culture medium broth Medium 523 by Kado and Heskett. Then, all phytopathogenic bacteria were cultured in a solid culture medium of Kado and Heskett's Medium 523 and incubated at a temperature of 28 ºC in a bacteriological oven and in aerobiosis, for 24 to 72 hours to confirm the purity of the strains. After confirmation, the strains were then used for assays to determine the minimum inhibitory concentration (MIC). The samples used for the broth microdilution technique to determine the minimum inhibitory concentration (MIC) were diluted in dimethylsulfoxide (DMSO), with the aid of an ultrasound bath. DMSO was used to carry out the dilutions. After all, it is considered the best solvent because, in addition to adequately solubilizing the samples, it is not very volatile, which does not allow sample precipitation before it is diffused. Samples were evaluated at concentrations between 1 and 400 µg/ mL. In one of the holes of each plate, the culture control was performed, and in the other, the sterility control of the broth (Medium 523 by Kado and Heskett). The microplates were incubated at 28ºC for 24 to 72 hours. Subsequently, 30 µL of 0.02% resazurin aqueous solution (Sigma) were added to each well, as a microbial growth indicator, and a change in blue color (without bacterial growth) or pink color (with bacterial growth) was observed.

The culture medium used to determine the antibacterial activity by the broth microdilution method to determine the minimum inhibitory concentration (MIC) was described by Kado and Heskett (1970). For its preparation, the compounds were dissolved in 1000 mL of distilled water. After that, homogenization and autoclaving occurred for 15 minutes at 121ºC. After sterilization by humid heat, 25.0 mL of this mixture was added to each Petri dish. Plates were incubated at 28°C for 24 to 72 hours. Subsequently, 30 µL of 0.02% resazurin aqueous solution were added to each well, and the MIC values were determined by visual reading after development with resazurin, which is an oxide-reduction indicator that has been used to assess the viability of microbial cells (MONTEJANO et al, 2005). Assays were performed in triplicate.

2.2.3 Evaluation of the time of death for bacteria (“time-kill curve”)

The kinetics of bactericidal activity (survival curve) was evaluated by determining the time of death of the sample against *Xanthomonas axonopodis* pv. For this, fresh cultures of the microorganism were diluted to a concentration of 1.0 X 10^5 CFU/mL and resuspended in saline with phosphate buffer (PBS). After adding the samples to their respective MIC, aliquots were removed at different times (initially 0, 2, 6, 9, 24 and 48 hours) and placed in plates containing BHI (Brain Heart Infusion) medium for colony counting. The entire procedure was performed in triplicate and submitted to statistical analysis. (HWANG et al., 2004).

2.3 EVALUATION OF USNIC 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 (FRANKEN, 2006). 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 usnic 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

3.1 RESULTS OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE ISOLATED COMPOUNDS AGAINST PHYTOPATHOGENIC BACTERIA

To evaluate the efficiency of the evaluated substances, the same standard of the tests with fungi was used, since Holetz et al., (2002) cite the MIC values for antimicrobial activity, being, therefore, valid for both fungi and bacteria. Remembering that according to Holetz et al. (2002), in the evaluation of antimicrobial activity in the determination of the minimum inhibitory concentration (MIC) of natural products, we consider MIC values lower than 100 µg/mL present good antimicrobial activity, MIC >100 to 500 µg/mL present moderate antimicrobial activity, MIC values >500 to 1000 µg/mL show poor antibacterial activity, and MIC values greater than 1000 µg/mL are considered inactive.

Thus, we observed that the results of usnic acid, rosmarinic acid and difractaic acid were more promising against bacteria than against fungi (CINTRA et al.,2022). This result was expected, since bacteria are naturally more sensitive to antimicrobial agents than fungi (OSTROSKY, 2008).

Among usnic acid, rosmarinic acid and difractaic acid, the most promising was usnic acid. It showed good antibacterial activity against all evaluated species (MIC values lower than 100 µg/mL), except for *Burkholderia cepacia*, whose MIC value was 400 µg/mL.

Usnic acid was more effective against strains *Xanthomonas axonopodis* pv and *Xanthomonas campestris* pv with MIC values of 3.12 µg/mL and 6.25 µg/mL, respectively. Followed by *Pantoea ananatis* and *Curtobacterium flaccumfaciens* with MIC values of 12.5 µg/mL. Later against *Clavibacter michiganensis*, *Pseudomonas syringae* pv, *Streptmyces scabiei*, *Ralstonia solanacearum* and *Burkholderia cepacia* with MIC values of 25 µg/mL, 50 µg/mL, 50 µg/mL, 100 µg/mL and 400 µg/mL, respectively.

Rosmarinic and difractaic acids, despite showing less efficiency compared to usnic acid, also showed promise in relation to the evaluated bacteria. Its results range from good to moderate antibacterial activity.

Rosmarinic acid had its lowest MIC value compared to *Ralstonia solanacearum* with a MIC of 25 µg/mL, and the difractaic acid showed a MIC value of 50 µg/mL against the same species. Table 2 shows the results of the antimicrobial activity of the isolated compounds against phytopathogenic bacteria.

Tabela 2: MIC results (µg/mL) of the isolated compounds against phytopathogenic bacteria.

	MIC (µg/mL)		
Microorganisms	**Rosmarinic acid**	**Usnic acid**	**Difractaic acid**
Xanthomonas axonopodis pv	400	3.12	200
Xanthomonas campestris pv	>400	6.25	200
Clavibacter michiganensis	>400	25	400
Pantoea ananatis	400	12.5	200
Burkholderia cepacia	>400	400	400
Ralstonia solanacearum	25	100	50
Pseudomonas syringae pv	100	50	100
Streptmyces scabiei	400	50	200
Curtobacterium flaccumfaciens	100	12,5	400

The MIC results of the control strains *Echerichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 was 0.7375 and 0.3688 µg/mL against tetracycline. The results found are in line with those described by CLSI, ensuring that the technique was performed correctly.

Xanthomonas axonopodis pv. passiflora is a gram-negative phytopathogenic bactéria and according to Nisengard & Newman, (1994), Gram-negative bacteria have an outer membrane with a trilaminar structure, that is, a thin inner peptidoglycan layer, a lipoprotein layer and a lipopolysaccharide layer. In contrast, Gram-positive bacteria have a simpler cell wall, consisting of a single thick layer of peptidoglycan. It can be suggested that the differences in activity found for the compounds in this work against Gram-positive and Gram-negative bacteria may be due to structural differences between them. It should be noted that the results obtained against Gram-negative bacteria in the present study were promising for some of the evaluated compounds.

Alves et al. (2008) in a comparative study of three screening techniques, concluded that the broth microdilution test (MIC) was the best option to assess antimicrobial activity. This result corroborates the choice of test for this work.

If we consider that the agricultural pesticides currently on the market are not specific, that is, the use of natural products would be a great advance in the control and management of diseases caused by these phytopathogenic bacteria. And, above all, rural producers would be recommended a specific product that is certainly less harmful 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 (RIBEIRO-SANTOS et al., 2017).

There are several categories of secondary metabolites with possible antimicrobial action in plant extracts, including terpenes, phenols and nitrogenous compounds (AGOSTINI-COSTA, 2012). Additionally, the possibility of synergism between these molecules can increase the antimicrobial efficiency of different types of plants. In view of the results obtained, the contribution of some of the selected compounds in the present study to the search for new products to control phytopathogenic bacteria was evidenced. The control of bacterial diseases in plants is still a challenge, mainly due to the limited availability of bactericides (LACOBELLIS et al, 2005). As a result, several strategies have been adopted in the control and management of bacterial diseases, such as the use of pathogen-free seeds and seedlings, the use of copper-based formulations and the use of resistant cultivars (QUEZADO & LOPES, 2010).

3.2 SURVIVAL CURVE OF THE BACTERIUM *XANTHOMONAS AXONOPODIS* PV TREATED WITH USNIC ACID

Figure 2 shows the results of the treatment of the *Xanthomonas axonopodis* pv with the usnic acid. It appears that at a concentration of 3.12 µg/mL, after 48 hours, usnic acid was bactericidal for the studied species. Table 3 shows the averages of the results obtained.

Gao et al., (2017) would evaluate the antibacterial activity of the compound Fubianezuofeng against *Xanthomonas axonopodis*, and as in this experiment, the bacteria showed sensitivity to the component.

Moom et al. (2020) identified that chitosan has antibacterial activity, through tests carried out against *Xanthomonas axonopodis*. Like them, Malamud et al. (2011), Ballottin et al. (2017) and Mottadi et al. (2018), also identified compounds with antibacterial potential against *Xanthomonas axonopodis* .

Thus, it is believed that it may present some particularity that makes it more sensitive when compared to the other evaluated microorganisms.

Figure 2. Survival curve of the bacterium *Xanthomonas axonopodis* pv treated with usnic acid.

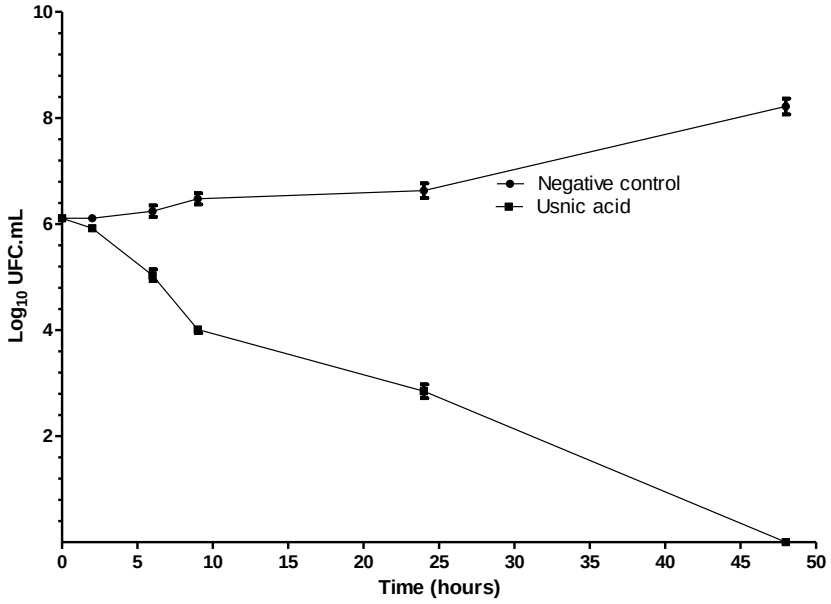


Table 3: Bacterial Kinetics with Usnic Acid against *Xanthomonas axonopodis pv*

Time (h)	CFU count/mL	Log 10	Log 10	Log 10
0	$6.5x10^4$	6.11	6.11	6.11
2	$4.3x10^4$	5.93	5.98	5.85
6	$5.3x10^3$	5.02	5,15	4.93
9	$5.1x10^2$	4.00	3.95	4.08
24	$3.9x10^1$	2.89	2.70	2.95
48	0.0	0.00	0.00	0.00

3.3 RESULTS OF THE EVALUATION OF THE CYTOTOXIC ACTIVITY OF USNIC ACID IN GM7492A CELLS (HUMAN FIBROBLASTS)

The results obtained in the antiproliferative activity assay are shown in table 4, where the curve represents the average of three independent experiments, and the error bar represents the standard deviation. The results revealed that there were no significant differences between the cultures treated with usnic acid (15.6µg/ml to 2000.0µg/ml) and the negative control, evidencing the absence of cytotoxic effects at the tested concentrations.

Table 4. Cell viability (%) after exposure of GM7492A (human fibroblasts) to different concentrations of usnic acid (15.6µg/ml to 2000.0µg/ml).

NC	SC	2000	1000	500	250.0	125.0	62.5	31.3	15.6
98.46	97.11	85.85	94.24	92.05	96.73	94.69	99.37	101.79	95.52
97.63	96.95	96.43	96.35	99.75	99.45	97.41	96.65	97.63	103.83
103.9	95.90	89.71	91.22	88.35	98.54	96.80	99.45	106.47	105.11

NC: negative control; SC:solvent control

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REFERENCES

AGOSTINI-COSTA TS, BIZZO VRF, SILVEIRA HR, GIMENES D. Secondary Metabolites. In: Dhanarasu S. (Ed.) **Chromatography and Its Applications**. *Rijeka: Croatia: Intech*: 131-164, 2012

ALVES, E.G.; VINHOLIS, A.H.C.; CASEMIRO, L.A.; FURTADO, N.A.J.C.; ANDRADE E SILVA, M.L.; WILSON ROBERTO CUNHA, W.R; MARTINS, C.H.G. Estudo comparativo de técnicas de *screening* para avaliação da atividade antibacteriana de extratos brutos de espécies vegetais e de substâncias puras. **Química Nova**, 31, 1224-1229, 2008

BALLOTTIN, Daniela et al. Antimicrobial textiles: Biogenic silver nanoparticles against *Candida* and *Xanthomonas*. **Materials Science and Engineering: C**, v. 75, p. 582-589, 2017.

CARVALHO, Sheila Salles de et al. Efeito inseticida sistêmico de nanoformulações à base de nim sobre *Bemisia tabaci* (*Hemiptera: Aleyrodidae*) biótipo B em tomateiro. **Bragantia**, v. 74, n. 3, p. 298-306, 2015.

CELOTO, Mercia Ikarugi Bomfim et al. Atividade antifúngica de extratos de plantas a *Colletotrichum gloeosporioides*. **Acta Scientiarum. Agronomy**, v. 30, n. 1, p. 1-5, 2008.

Cintra, L. S.; Tozatti, M. G.; Vasconcelos, M. A. L.; MARTINS, C. H. G.; SILVA, M. L. A.; JANUARIO, A. H.; Pauletti, P. M.; CUNHA, W.R. Atividade antimicrobiana de substâncias isoladas de *Usnea steineri* frente a fitopatógenos In: **O papel fundamental da Química entre as Ciências Naturais**, 1a. ed., Ponta Grossa -PR: Atena Editora, v.1, p. 146-159. 2022

CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*; Approved Standard, 8th edn, M7-A8. Wayne, PA: **Clinical and Laboratory Standards Institute**, 2009.

FERREIRA, Fátima Teresinha Rampelotti; VENDRAMIM, José Djair; FORIM, Moacir Rossi. Bioatividade de nanoformulações de nim sobre a traça-do-tomateiro. **Ciência Rural**, v. 42, n. 8, p. 1347-1353, 2012.

FONSECA, Maira Christina Marques et al. Potencial de óleos essenciais de plantas medicinais no controle de fitopatógenos. **Revista Brasileira de Plantas Medicinais**, v. 17, n. 1, p. 45-50, 2015.

FRANKEN, Nicolaas AP et al. Clonogenic assay of cells in vitro. **Nature protocols**, v. 1, n. 5, p. 2315-2319, 2006.

GAO, Manni et al. Label-free quantitative proteomic analysis of inhibition of *Xanthomonas axonopodis* pv. citri by the novel bactericide Fubianezuofeng. **Pesticide Biochemistry and Physiology**, v. 138, p. 37-42, 2017.

GHINI, Raquel; KIMATI, Hiroshi. **Resistência de fungos a fungicidas**. Jaguariúna: Embrapa Meio Ambiente, 2000., 2000.

HOLETZ, Fabíola Barbiéri et al. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. **Memórias do Instituto Oswaldo Cruz**, v. 97, n. 7, p. 1027-1031, 2002.

HONDA, N. K. Liquens de Mato Grosso do Sul – Estudo químico e avaliação da atividade biológica. **Tese de Doutorado** – Universidade Estadual Paulista – UNESP, Araraquara, SP. 1997.

HWANG, Jae-Kwan et al. Isopanduratin A from *Kaempferia pandurata* as an active antibacterial agent against cariogenic *Streptococcus mutans*. International **Journal of Antimicrobial Agents**, v. 23, n. 4, p. 377-381, 2004.

KADO, C. I. et al. Selective media for isolation of agrobacterium, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. **Phytopathology**, v. 60, n. 6, p. 969-976, 1970.

KHUNT, M.; RIMPLER, H.; HEINRICH, M. Lignans and other compounds from the Mixe Indian medicianl plant *Hyptis verticillata*. **Phytochemistry,** v. 36, p. 485-489, 1994.

KÖNIG G.M., WRIGHT A.D. (**1999**) ^{1}H and ^{13}C–NMR and biological activity investigations of four lichen–derived compounds. **Phytochemical Analysis**,*10*, 279–284.
LACOBELLIS, N.S.; CANTORE, P.L.; FRANCESCO CAPASSO, F.; FELICE SENATORE, F. Antibacterial activity of *Cuminum cyminum* L. and *Caru carvi* L. essential oils. **Journal of Agricultural and Food Chemistry**, *53*, 57−61, 2005.

MA, Ya-Tuan et al. Natural products as sources of new fungicides (I): Synthesis and antifungal activity of acetophenone derivatives against phytopathogenic fungi. **Chemical Biology & Drug Design**, v. 81, n. 4, p. 545-552, 2013.

MALAMUD, Florencia et al. The *Xanthomonas axonopodis pv. citri* flagellum is required for mature biofilm and canker development. **Microbiology**, v. 157, n. 3, p. 819-829, 2011.

MDEE, Ladislaus Kakore; MASOKO, Peter; ELOFF, Jacobus Nicolaas. The activity of extracts of seven common invasive plant species on fungal phytopathogens. **South African Journal of Botany**, v. 75, n. 2, p. 375-379, 2009.

MOON, Chaeyeong et al. Antibacterial activity of various chitosan forms against *Xanthomonas axonopodis pv.* glycines. **International Journal of Biological Macromolecules**, v. 156, p. 1600-1605, 2020.

MOTTADI, Lakshmi Sri; DEVARA, Sandhya Deepika; SHAIK, Taher Ibrahim. Anti-microbial activity of some Botanicals against the *Xanthomonas axonopodis pv punicae* in Pomegranate. **Journal of Pharmacognosy and Phytochemistry**, v. 7, n. 4, p. 2812-2815, 2018.

NAGHETINI, C.C. Caracterização físico-química e atividade antifúngica dos óleos essenciais da cúrcuma. **Dissertação** (Mestrado em Ciências de alimentos). Farmácia da UFMG, 2006.

NISENGARD RJ, Newman MG. **Oral Microbiology and Immunology**, 2nd ed. Philadelphia, PA: Sauders, 477,1994.

OSTROSKY, Elissa A. et al. Métodos para avaliação da atividade antimicrobiana e determinação da concentração mínima inibitória (CMI) de plantas medicinais. **Revista Brasileira de Farmacognosia**, v. 18, n. 2, p. 301-307, 2008.

PASTRO, D.C.; PASCUALI, L.C.; SANDRI, D.O.; ZELA, S.P.; SILVA, F.S. Diagnóstico de extratos vegetais com potencial para o controle fúngico. **Enciclopédia Biosfera**, 8, 14, 389-396, 2012.

QUEZADO-DUVAL., A.M.; LOPES, C.A. Mancha bacteriana: uma atualização para o sistema de produção integrada de tomate indústria. Brasília, DF: Embrapa Hortaliças**. Embrapa Hortaliças.** ***Circular Técnica****, 84*, 28p. 2010.

RIBAS, P. P.; MATSUMURA, A. T. S. A química dos agrotóxicos: impacto sobre a saúde e meio ambiente. **Revista Liberato**, v. 10, n. 14, p. 149-158, 2009.

RIBEIRO-SANTOS R, ANDRADE M, MELO NR, SANCHES-SILVA A. Use of essential oils in active food packaging: recent advances and future trends. ***Trends Food Sci Technol***.61:132-140, 2017.

SALES, Maria Diana Cerqueira et al. Antifungal activity of plant extracts with potential to control plant pathogens in pineapple. **Asian Pacific Journal of Tropical Biomedicine**, v. 6, n. 1, p. 26-31, 2016.

SOUZA, A.E.F.; ARAÚJO, E.; NASCIMENTO, L.C. Atividade antifúngica de extratos de alho e capim-santo sobre o desenvolvimento de *Fusarium* isolado de grãos de milho. **Fitopat. Brasil**. 32,6,465-471,2007.

TOZATTI, Marcos G. et al. The activity of the *Lichen Usnea steineri* and its Major Metabolites against Gram–positive, Multidrug–resistant Bacteria. **Natural Product Communications**, v. 11, n. 4, p. 493-496, 2016.

VELLUTI, A. et al. Impact of essential oils on the growth rate, zearalenone and deoxynivalenol production by *Fusarium graminearum* under different temperature and water activity conditions in maize grain. **Journal of Applied Microbiology**, v. 96, n. 4, p. 716-724, 2004.