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Short Communication

***In vitro* antimicrobial activity, total polyphenols and flavonoids contents of *Nopalea cochenillifera* (L.) Salm-Dyck (Cactaceae)**Raquel M. M. NECCHI^{1*}, Izabel A. ALVES¹, Sydney H. ALVES², Melânia P. MANFRON³

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This study evaluated the antimicrobial activity *in vitro* qualitative and quantitative methods, and made the determination of total polyphenols and flavonoids in the ethanol extract of *Nopalea cochenillifera*. The assessment determined the antimicrobial minimum inhibitory concentration (MIC) against *Escherichia coli*, *Salmonella typhi*, *Micrococcus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Candida albicans*, *Candida glabrata*, *Prototheca zopffi*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* e *Malassezia furfur*. The determination of total polyphenols and flavonoids were significant when compared respectively to the standards of gallic acid and rutin.

Key words: Antimicrobial, Cactaceae, flavonoids, *Nopalea cochenillifera*, polyphenols

The constant administration of antifungal and antibacterial becomes inefficient conventional treatment of mycosis and bacterial infections, providing resistance of microorganisms and difficult to cure patients. Many researchers have studied the possibility of finding biological activities, among them the antimicrobial plants for new compounds or substances that may be effective in drug therapy (Cole, 1994).

Nopalea cochenillifera is a shrubby plant belonging to the family Cactaceae, known as "palma-doce" or "palma-miúa". It is used in traditional medicine as anti-inflammatory, analgesic, diuretic, hypoglycemic (Park et al., 2001, Cetto, 2005) for the treatment of hypertension and kidney stones (Lans, 2006). Studies *in vitro* *N. cochenillifera* showed significant antimicrobial activity against *Escherichia coli*, *Salmonella enterica*, and *Candida albicans* (Gomez-Flores et al., 2006). The cladodes of *N. cochenillifera* verified the presence of flavonoids, tannins, saponins and anthraquinones, and highlighted the occurrence of β -sitosterol (Necchi et al., 2010; Gomez-Flores et al., 2006). It has also been envisaged to inhibit the virus herpes simplex type I (Szuchman, 1999).

Among the many different uses and chemical composition of the popular medicinal plant this study aimed to investigate the antibacterial and antifungal activity in vitro ethanol extract of *Nopalea cochenillifera* against *Escherichia coli*, *Salmonella typhi*, *Micrococcus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Prototheca zopffi*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Malassezia furfur*. Like, make the determination of total polyphenols and flavonoids.

Materials and Methods

Collection of botanical material and obtaining the plant extract

The cladodes of *Nopalea cochenillifera* (L.) Salm-Dyck were collected in Santa Maria, Rio Grande do Sul, Brazil. A sample of plant material was identified and deposited in the Herbarium of Biology Department of UFSM as voucher specimen SMDB 11.835. The ethanol extract of fresh cladodes of *N. cochenillifera* was obtained by cold maceration and reduced to dry.

Antimicrobial activity by bioautography

The antimicrobial activity was performed by bioautography as Hamburger and Cordell (1987) and Rahalison et al., (1991) using the indicator microorganisms American Type Culture Collection (ATCC) *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* and *Candida albicans*. Amoxicillin was used and Nystatin as standard antibacterial and antifungal. The inoculations were prepared in saline, with transmittance of 25% and added to Mueller Hinton agar (bacteria) in the proportion of 0.5% and Sabouraud Dextrose agar (fungi) at 2%. Petri dishes containing the chromatograms developed a chromatographic silica gel were incubated for 18 hours at 35°C for bacteria and for 48 hours at 25°C for fungi. After this period they were sprayed with chloride 2,3,5-triphenyl tetrazolium to 2%, and at 2 h incubation allowing the detection of halos of inhibition.

Antimicrobial activity by microdilution

The completion of the antimicrobial activity was performed according to the protocols of the CLSI M27A3 (2008) and M7A4 (1987) for the testing of antibacterial and antifungal activity respectively, thereby determining the minimum inhibitory concentration (MIC).

Preparation of microdilution plates

For the tests were used microdilution plates with a cavity whose capacity is 500 µl, arranged in 8 rows and 12 columns, totaling 96 wells. The plates and their lids were first washed with a hypochlorite solution at 10%. Next, plates and lids were dried at 32 °C. After drying, were placed in a laminar flow hood under ultraviolet light-C for 2 hours for sterilization. After sterilization, the plates were wrapped in paper and stored until the time they are used.

Preparation of inoculum

The yeasts were inoculated in tubes with Sabouraud agar and incubated at 30 °C for 24 hours. We subsequently prepared a suspension of microorganisms in sterile 0.85% saline, adjusting the turbidity according to the scale (0.5) for MacFarland. The preparation of the inoculum of the bacterial species was performed similarly, by replacing only the means of watering by watering Sabourand and YNB BHI and Mueller-Hinton broth, respectively

Inoculation into culture medium

Each well of the microdilution plate already containing 0.1 ml of the test drug concentration was inoculated with 0.1 ml suspension of microorganism. The cavity of the positive control contained 0.1 ml of inoculum and 0.1 ml of medium without the drug test. After inoculation, the plate was closed, identified and inoculated as time period recommended by the methodology for each microorganism. The test was performed in duplicate. The concentrations of the extract ranged from 10 to 0.156 mg / ml for bacteria and 5 to 0.078 mg / ml for fungi.

The microorganisms used in the study were: *Escherichia coli* ATCC 8739, *Salmonella typhi* ATCC 9120, *Micrococcus* ATCC 9341, *Klebsiella pneumoniae* ATCC 10031, *Staphylococcus aureus* ATCC 25923, *Candida albicans* 40175, *Candida glabrata* ATCC 90030, *Prototheca zopffi* ATCC 461, *Cryptococcus neoformans* ATCC 28952, *Saccharomyces cerevisiae* ATCC 9763 e *Malassezia furfur*.

Incubation and reading test

The microplates inoculated with bacteria were incubated at 35 °C for 24 hours, and inoculated with yeasts were incubated at 30 °C for 48 hours, or until the growth of the positive control could be evidenced.

The reading was performed after incubation by visual composition with the control of positive growth, determined as MIC the lowest concentration of the substance was not found where growth of the microorganism.

Dosage of polyphenols

The dosage of polyphenols was performed by the modified Folin-Ciocalteu (Chandra and Mejia, 2004). The extracts of *N. cochenillifera* were dissolved in water at a concentration of 0.04% in a 1 ml. Soon, it was added the same volume of Folin-Ciocalteu, await 5 minutes, add 2 ml of 20% Na₂CO₃ and it is expected 20 minutes. After it holds reading at 730 nm in UV/VIS spectrophotometer to obtain the concentration of total polyphenols present. The analysis will be performed in the dark and in triplicate.

Absorbance data of samples were compared with the standard curve obtained from solutions with increasing concentrations of gallic acid. The standard solution of gallic acid was prepared with distilled water in a concentration at concentrations 4 µg/ml, 6µg/ml , 10µg/ml , 20µg/ml , 30 µg/ml, 40µg/ml e 50µg/ml. The equation obtained for the calibration curve of gallic acid was $Y = 0,0272 x - 0,0953$ ($r = 0,9981$).

Dosage of flavonoids

The dosage of flavonoids was carried out according to Rio (1996) modified using rutin as a standard solution of aluminum chloride in methanol.

The extract of *N. cochenillifera* was diluted in 70% methanol at a concentration of 0.4%. To 1.1 ml sample was added to 75 µl of aluminum chloride to 5% methanol and 3.9 ml of 70 %. After a relaxing 30-minute reading was done at 425 nm on UV/VIS and obtained the concentration of flavonoids.

The analysis was performed in triplicate. The absorbance data of samples were compared with the standard curve obtained from solutions with increasing concentrations of rutin. The standard solution of rutin was prepared with 70% methanol at a concentration of 100 mg/ml. To obtain the solutions of the standard curve were used aliquots of standard solution, plus 75 µl of aluminum chloride to 10 ml and supplemented with 70% methanol. After resting for 30 minutes was done reading at 425 nm. The equation obtained for the calibration curve of rutin was $Y = 0,0026 + 0,0496 X$ ($r = 0,9988$).

Results

The ethanol extract of *N. cochenillifera* verified antimicrobial activity against Gram-positive cocci used in the tests of bioautography, *S. aureus* & *S. epidermidis*, observed by the presence of a small halo of growth inhibition around the region to extract migration along the chromatographic plate (Figure 1).

In the strains of Gram-negative (*E. coli*, *K. pneumoniae* & *P. aeruginosa*) and fungi (*S. cerevisiae* & *C. albicans*) tested there was no inhibition of growth rates.

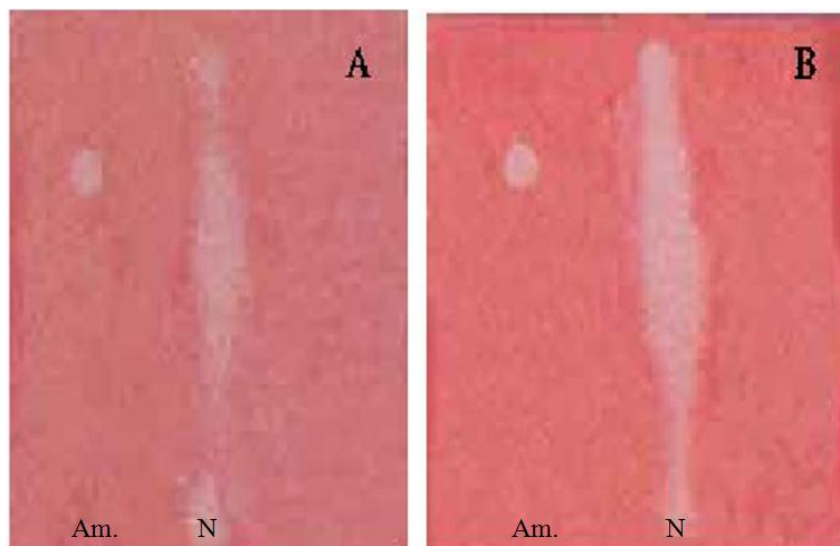


Figura 1 - Bioautograma the ethanol extract of cladodes of *N. cochenillifera* (N) and standard Amoxicillin (Am). A: *S. aureus*; B: *S. epidermidis*.

The results of antibacterial activity by broth microdilution method showed MIC of extract *Nopalea cochenillifera* 0,625 mg/ml to *Micrococcus*; 2,5 mg/ml to *S. aureus*; 5 mg/ml to *K. pneumoniae*; 5 mg/ml to *Salmonella typhi* and 10 mg/ml to *E. coli*. Tests to determine the antifungal activity of the extract showed MIC 2,5 mg/ml to *C. glabrata*; 0,625 mg/ml to *C. albicans*; 1,25 mg/ml to *Prototheca zopffi*; 1,25 mg/ml to *C. neoformans*; 1,25 mg/ml to *Saccharomyces cerevisiae* and 2,5 mg/ml to *Malassezia furfur* (Table 1).

Table 1. Minimum inhibitory concentration (MIC) of ethanol extract of *N. cochenillifera* against the tested microorganisms

Microrganismo testado	CIM (mg/mL)
<i>Micrococcus</i>	0,625
<i>S. aureus</i>	2,5
<i>K. pneumoniae</i>	5
<i>Salmonella typhi</i>	5
<i>E. coli</i>	10
<i>C. glabrata</i>	2,5
<i>C. albicans</i>	0,625
<i>Prototheca zopffi</i>	1,25
<i>C. neoformans</i>	1,25
<i>Saccharomyces cerevisiae</i>	1,25
<i>Malassezia furfur</i>	2,5

The result of the analysis indicate that the ethanol extract of *N. cladodes cochenillifera* presents, respectively, a content $29.62\% \pm 1,356$ and total polyphenol gallic acid equivalents / g. Regarding the analysis of flavonoids in the extract percentage $7.63\% \pm 0,075$ flavonoid rutin equivalents / g extract (Figure 2).

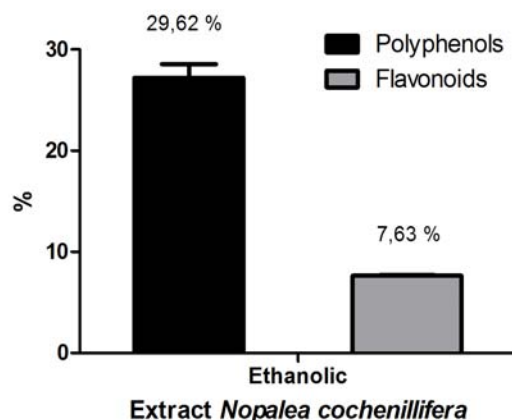


Figure 2. Determination of total content of total polyphenols and flavonoids from the ethanol extract of *N. cochenillifera*. Mean \pm SEM.

Discussion

Plant extracts are considered potentially good show inhibitory activity at concentrations as being $100\ \mu\text{g/ml}$ - $1000\ \mu\text{g/ml}$, being superior to traditional antibiotics and antifungals (0.01 to $10\ \mu\text{g/ml}$) (Fabry et al 1998; Tegos, 2002, Dall' Angol et al., 2003; Tanaka et al, 2005).

So according to this pattern established an ethanol extract of *N. cochenillifera* best result for *Micrococcus* & *C. albicans*, where the MIC was $625\ \mu\text{g/ml}$. According Gomez-Flores et al., (2006), the ethanol extract of fresh *Nopalea* showed antimicrobial activity by the colorimetric method in liquid medium, compared to *C. albicans* at concentrations as low as $3.9\ \mu\text{g/ml}$, and $62.5\ \mu\text{g/ml}$ for *S. enterica var.*, which shows a potent activity.

The ethanol extract of leaves of *Luheae divaricata* by the same method presented in this study, MIC of $750\ \mu\text{g/ml}$ against *Candida albicans* and *K. pneumononiae*; and $1500\ \mu\text{g/ml}$ against *E. coli* (Muller, 2006). The chemical composition of the extracts is due to the chemical solvent and method used to obtain, usually the active compounds are diluted and at low concentrations (Rates, 2001). Tanaka et al., (2005) estimated that the polar fractions of ethanol extract of leaves of *Luheae divaricata* have flavonoids, tannins with antimicrobial properties.

The bioautography is considered an efficient and sensitive assay for the determination of antimicrobial activity, as less than $2.5\ \text{mg}$ of substance so visible inhibition zone (Hamburger and Cordel, 1987). Magalhães et al., (1998) used bioautography tests with extracts of flavonoids isolated from roots of *Lonchocarpus montanus*, confirming the evidence of the test. Flavonoids are compounds that have many medicinal properties, among them the antimicrobial, antioxidant and antiinflammatory (Harborne and Willians, 2000).

Many studies show that plant extracts containing flavonoids, triterpenes and steroids have significant antimicrobial activity against several strains such as *Staphylococcus aureus*, *Streptococcus faecalis* and *Escherichia coli* (Baez et al., 1999; Xu and Lee, 2001; Chattopadhyay et al., 2001).

Rauha et al., (2000) conducted an antimicrobial screening of 13 phenolic compounds by diffusion methods against *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*, *Escherichia*

coli, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Staphylococcus nervous*, *Staphylococcus epidermidis* and found that quercetin and naringenin inhibited the growth of these organisms. Necchi et al., (2010) using histochemical analysis verified the presence of flavonoids in the region of hypodermic cladodes of *N. cochenillifera*. The content of polyphenols and flavonoids significant, seen in this study are similar and justify the popular use of this medicinal plant. The ethanol extract of *Acacia podalyriifolia* presented polyphenol content 29.04%, the same methodology of this study, this plant is considered with significant antioxidant activity (Andrade, 2007).

Conclusion

The ethanol extract *Nopalea cochenillifera* showed good antibacterial activity and showed an inhibitory activity against microorganisms tested. The content of polyphenols and flavonoids in *N. cochenillifera* help protect the body against the harmful action of free radicals acting in the oxidative process.

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