In vitro Antibacterial and Antifungal Activities of *Nopalea cochenillifera* Pad Extracts

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Abstract: Effectiveness of botanical treatments has been recognized by many, but scientific validation on the beneficial use of plants is scarce. Nopalea pads are probably native to Mexico or Central America and are used for human consumption and for medicinal purposes. In this study, in vitro antibacterial and antifungal activities of fresh and dried *Nopalea cochenillifera* (NC) were investigated. MICs of fresh NC hexanic, chloroformic and ethanolic fractions against *C. albicans* were 250, 250 and 3.9 µg mL^{-1} respectively; MICs of fresh NC against *Salmonella enterica* var. thyphimurium were 15.6, 62.5 and 3.9 µg mL^{-1} respectively; and MICs of fresh NC against Escherichia coli were 500, NA (no activity) and 3.9 µg mL^{-1} respectively. In addition, MICs of dried NC hexanic, chloroformic and ethanolic fractions against *C. albicans* were 31.2, 31.2 and 3.9 µg mL^{-1} respectively; MICs of dried NC against *Salmonella enterica* var. thyphimurium were 15.6, NA and 3.9 µg mL^{-1} respectively; and MICs of dried NC against *Escherichia coli* were NA, 62.5 µg mL^{-1} and NA respectively. The relevance of using fresh versus dried *Nopalea cochenillifera* pad extracts against in vitro microbial growth is discussed.

Key words: *Nopalea cochenillifera*, methanolic extract, hexanic extract, ethanolic extract, *in vitro*, antibacterial activity, antifungal activity, *Salmonella enterica*, *E. coli*, *Candida albicans*

INTRODUCTION

Nopalea tender young pads, also known as "nopalitos verdes", are probably native to Mexico or Central America. They are a good source of fruits ("tunas") and vegetables (nopalitos verdes) for human consumption, as well as fodder for cattle and other animals during the dry seasons. They are also used for medicinal purposes, in cosmetics, to produce dyes and as natural fences. These cacti belong to the subfamily Opuntioideae and comprise more than 200 species worldwide, 114 of which occur in Mexico[1-3]. They are commonly consumed as a fresh or cooked green vegetable in Mexico and in some parts of the United States[4,5]. In Mexico, annual production of this vegetal is about 600,000 tons, being the area of Milpa Alta, D.F. the most important producer. In the United States, *Nopalea cochenillifera* (L.) Salm-Dyck clone 1308 is produced in the States of California and Texas, with an annual production of 5,000 tons[6,7]. This species is for the most part free of spines and spine-hairs[8,9]. Cactus pads usually contain about 92% water, 4-6% carbohydrate, 1% protein, 0.2% fat, 1% minerals, vitamin C (12.7 mg/100g fresh weight) and ß-carotene (12.9 µg/ 100 g fresh weight)[10].

In addition to the hypoglycemic property of cacti[11], there are few reports on the biological activity of Nopalea species. These plants have been used to treat rheumatism and other inflammatory problems and diarrhea, as well as a diuretic and analgesic, particularly for ear and tooth aches[12-16]. The anti-inflammatory principle was related to a β-sitosterol[17]. *Nopalea cochenillifera* was also shown to inhibit herpes simplex virus type 1 infection[18]. Despite these biological properties, validation of any antibiotic potential against bacterial and fungal species has not been reported yet.

The present study was undertaken to evaluate the effects of the methanolic, hexanic and ethanolic fractions of *Nopalea cochenillifera* pads on in vitro antimicrobial activity against *Escherichia coli*, *Salmonella enterica* var. thyphimurium and *Candida albicans*.

MATERIALS AND METHODS

Reagents, culture media and microbial strains: Sodium dodecyl sulfate (SDS), N,N-dimethylformamide (DMF) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). *Candida albicans* (ATCC 32354) and *Escherichia coli*

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(ATCC 25922) were purchased from the American Type Culture Collection (Rockville, MD) and *Salmonella enterica* var. thyphimurium was a food-borne native isolate. Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37°C in a solution of 50% each DMF and demineralized water and the pH was adjusted to 4.7.

Preparation of *Nopalea cochenillifera* extract: The cactus material used in this study was identified as *Nopalea cochenillifera*, commonly known as "nopal verdura", by Marcela González-Vargas, Chief of the Herbarium of the Biological Sciences College at Autonomous University of Nuevo Leon, with a voucher specimen # 024189 (UAN). It was obtained from a plantation in Villa de García, Nuevo León, located northeast of México. Fresh (not dried) *Nopalea cochenillifera* pads were cut in small pieces (1000 g) and were macerated in an Erlen Meyer flask with 3 liters of hexane, chloroform or ethanol for 7 days each at room temperature. After this, extracts were filtered (Whatman filters, Whatman International Ltd., Maidstone, England) and evaporated in a rotary evaporator Büchi (model R-114, Brinkmann Instruments Inc., Switzerland) to dryness obtaining respectively, 3.47 g, 1.63 g and 19.8 g. In addition, dried pads were pulverized and 20 g were placed in 3 independent Soxhlet extractors (PYREX, model 3840-LCO, Mexico) using hexane, chloroform or ethanol. After this, extracts were filtered (125-mm diameter Whatman filters and evaporated in a rotary evaporator Büchi to dryness obtaining respectively, 5.7 g, 5.32 g and 30.31 g.

Microbicidal activity of *N. cochenillifera* extracts: We selected *Candida albicans*, *Escherichia coli* and *Salmonella enterica* var. thyphimurium because they are related to AIDS and gastrointestinal disorders. These strains were maintained and activated in specific culture media for each type of microorganism. We determined the percentage of microbial growth inhibition by Nopalea extract fractions in liquid medium by a colorimetric technique[19]. *Candida albicans* was activated by plating an aliquot in YM agar (Difco Laboratories, Detroit, Mich) for 24 h at 37°C[19]. Next, a loop of the culture was taken and suspended in YM broth (Difco, Becton Dickinson Microbiology Systems, Sparks, MD) and adjusted to 1×10^3 yeasts mL^{-1}. Bacteria were cultured in Mueller-Hinton agar (Becton Dickinson de México, Estado de México) for 24 h at 37°C, after which a single colony was taken and suspended in BHI broth (DIBICO, Co., pH 7.4, México DF) at a concentration of 2×10^3 bacteria mL^{-1}. Fifty microliters of the microbial suspensions were plated (in YM and BHI broths for yeasts and bacteria respectively) in flat-bottomed 96-well plates (Corning Incorporated, Corning, NY) in the presence or absence of serial dilutions (1:2) of the Nopalea extract (50 µL)

to be tested. Vehicle controls were also tested; they were shamed prepared and processed as if they contained Nopalea extracts. Plates were then incubated for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which the tetrazolium salt MTT was added to all wells at a final concentration of 0.5 mg mL^{-1} and plates were incubated for 4 additional hours. At the end of the incubation period, 50 µl of extraction buffer were added to all wells and plates were incubated overnight at 37°C. Optical densities resulting from dissolved formazan crystals, were then read in a microplate reader (Bio-Tek Instruments inc., EL-311, Winooski, VT) at 540 nm.

Statistical analysis: The results were expressed as mean $\pm$ SEM of triplicate determinations from three independent experiments. Statistical significance was assessed by the Student t test.

RESULTS

Antimicrobial activity of fresh *N. cochenillifera* hexanic fraction: As observed in Fig. 1, Nopalea hexanic fraction did not alter *C. albicans* or *E. coli* growth; any observed inhibitory effect was due to the vehicle. However, this fraction caused 30 to 37 percent *Salmonella enterica* var. thyphimurium growth inhibition at the concentrations tested, but only the activity at concentrations 15.6, 31.2 and 62.5 µg mL^{-1} (32.4, 29.6 and 29.8 percent growth inhibition respectively) were attributed to the effect of the fraction (P < 0.05) when comparing the effect of the hexanic fraction versus the vehicle control (Fig. 1); any other inhibitory effect was due to the vehicle.

Antimicrobial activity of fresh *N. cochenillifera* chloroformic fraction: As observed in Fig. 2, Nopalea chloroformic fraction did not alter *C. albicans* growth; any observed inhibitory effect was due to the vehicle. This fraction, however, caused 20 to 30 percent *Salmonella enterica* var. thyphimurium growth inhibition at the concentrations tested, but only the activity at concentrations 62.5, 125 and 250 µg mL^{-1} (23.6, 24.3 and 28.1 percent growth inhibition respectively) were attributed to the effect of the fraction (P < 0.05) when comparing the effect of the chloroformic fraction versus the vehicle control (Fig. 2); any other inhibitory effect was due to the vehicle. This fraction also caused 22 to 42 percent *E. coli* growth inhibition at the concentrations tested, but only the activity at 125 µg mL^{-1} (22.2 percent growth inhibition) was attributed to the effect of the fraction (P < 0.05) when comparing the effect of the chloroformic fraction versus the vehicle control (Fig. 2); any other inhibitory effect was due to the vehicle.

Antimicrobial activity of fresh *N. cochenillifera* ethanolic fraction: As observed in Fig. 3, Nopalea ethanolic fraction caused 20 to 31 percent *C. albicans* growth inhibition at concentrations ranging from
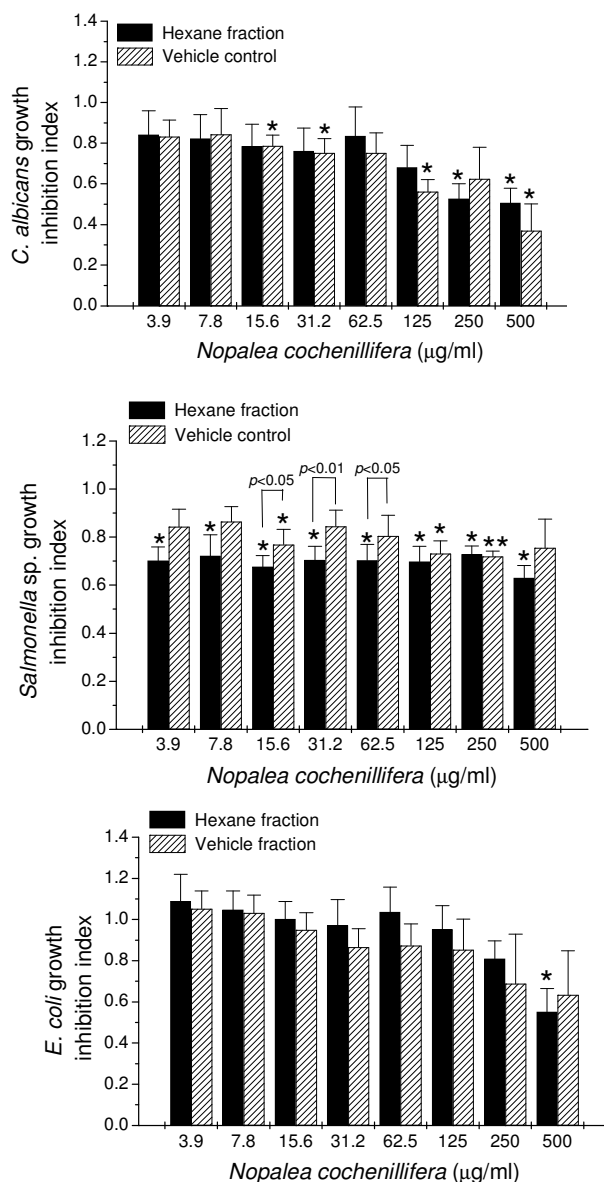
Fig. 1: Effect of fresh Nopalea hexanic fraction on *C. albicans*, *Salmonella enterica* and *E. coli* growth. *Candida albicans* was adjusted to 1 X 10³ yeasts mL⁻¹ in YM broth and bacteria were adjusted to 2x 10³ bacteria mL⁻¹ in ICC broth as explained in the text. Then bacteria and yeasts were cultured in the presence or absence of various concentrations of the Nopalea extract or vehicle controls, for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which MTT was added to all wells and plates were incubated for 4 additional hours. At the end of the incubation period, extraction buffer was added to all wells and optical densities were read at 540 nm as detailed in the text. Data represent means ± SEM of growth inhibition indexes of triplicates from 3 independent experiments. **, P < 0.01; *, P < 0.05 as compared with untreated control

Fig. 2: Effect of fresh Nopalea chloroformic fraction on *C. albicans*, *Salmonella enterica* and *E. coli* growth. *Candida albicans* was adjusted to 1 X 10³ yeasts mL⁻¹ in YM broth and bacteria were adjusted to 2x 10³ bacteria mL⁻¹ in ICC broth as explained in the text. Then bacteria and yeasts were cultured in the presence or absence of various concentrations of the Nopalea extracts or vehicle controls, for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which MTT was added to all wells and plates were incubated for 4 additional hours. At the end of the incubation period, extraction buffer was added to all wells and optical densities were read at 540 nm as detailed in the text. Data represent means ± SEM of growth inhibition indexes of triplicates from 3 independent experiments. *, P < 0.05 as compared with untreated control

3.9 to 250 μg mL⁻¹, but only the activity at concentrations 7.8, 15.6, 31.2 and 125 μg mL⁻¹ (23.2, 30.6, 21.6 and 24.7 percent growth inhibition respectively) were attributed to the effect of this fraction (P < 0.05) when comparing the effect of the ethanolic fraction versus the vehicle control (Fig. 3);
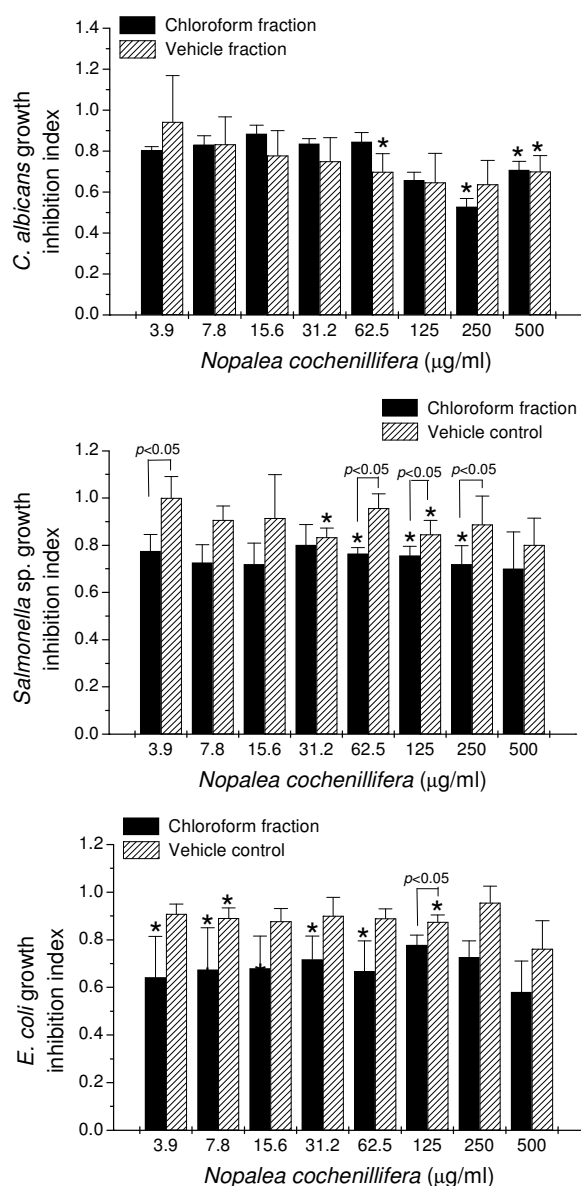
any other observed inhibitory effect was due to the vehicle. This fraction, however, caused 41 to 57 percent *Salmonella enterica* var. thyphimurium growth inhibition at the concentrations tested, but only the activity at 500 μg mL⁻¹ (57.2 percent growth inhibition) was attributed to the effect of the fraction (P < 0.05)
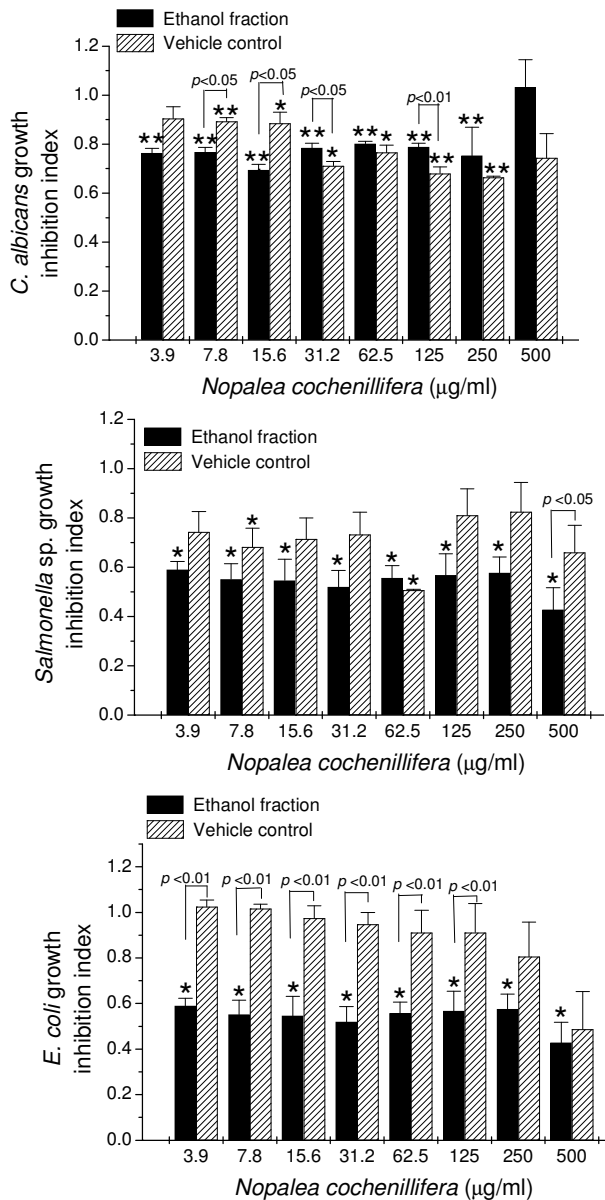
Fig. 3:   Effect of fresh Nopalea ethanolic fraction on *C. albicans*, *Salmonella enterica* and *E. coli* growth. *Candida albicans* was adjusted to 1 X 10^3 yeasts mL^{-1} in YM broth and bacteria were adjusted to 2x 10^3 bacteria mL^{-1} in ICC broth as explained in the text. Then bacteria and yeasts were cultured in the presence or absence of various concentrations of the Nopalea extracts or vehicle controls, for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which MTT was added to all wells and plates were incubated for 4 additional hours. At the end of the incubation period, extraction buffer was added to all wells and optical densities were read at 540 nm as detailed in the text. Data represent means ± SEM of growth inhibition indexes of triplicates from 3 independent experiments. **, P < 0.01; *, P < 0.05 as compared with untreated control

Fig. 4:   Effect of dried Nopalea hexanic fraction on *C. albicans*, *Salmonella enterica* and *E. coli* growth. *Candida albicans* was adjusted to 1 X 10^3 yeasts mL^{-1} in YM broth and bacteria were adjusted to 2x 10^3 bacteria mL^{-1} in ICC broth as explained in the text. Then bacteria and yeasts were cultured in the presence or absence of various concentrations of the Nopalea extracts or vehicle controls, for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which MTT was added to all wells and plates were incubated for 4 additional hours. At the end of the incubation period, extraction buffer was added to all wells and optical densities were read at 540 nm as detailed in the text. Data represent means ± SEM of growth inhibition indexes of triplicates from 3 independent experiments. **, P < 0.01; *, P < 0.05 as compared with untreated control

when comparing the effect of the ethanolic fraction versus the vehicle control (Fig. 3); any other inhibitory effect was due to the vehicle. This fraction did not alter *E. coli* growth (Fig. 3).
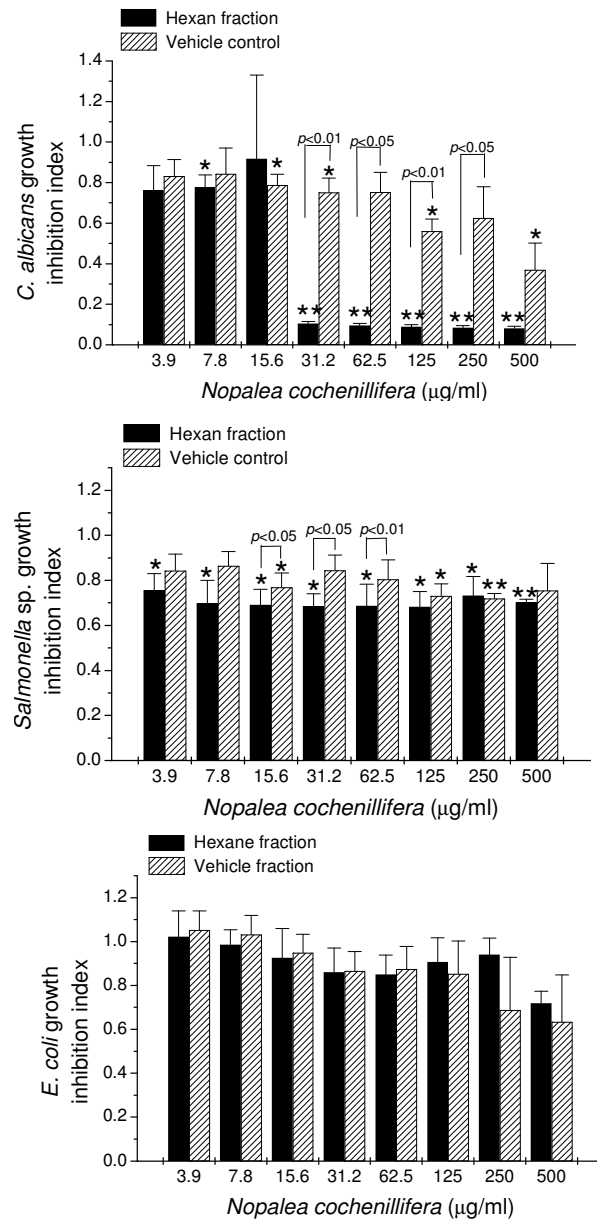
Antimicrobial activity of dried *N. cochenillifera* hexanic fraction: As observed in Fig. 4, Nopalea hexanic fraction caused 8 to 92 percent *C. albicans* growth inhibition at the concentrations tested,
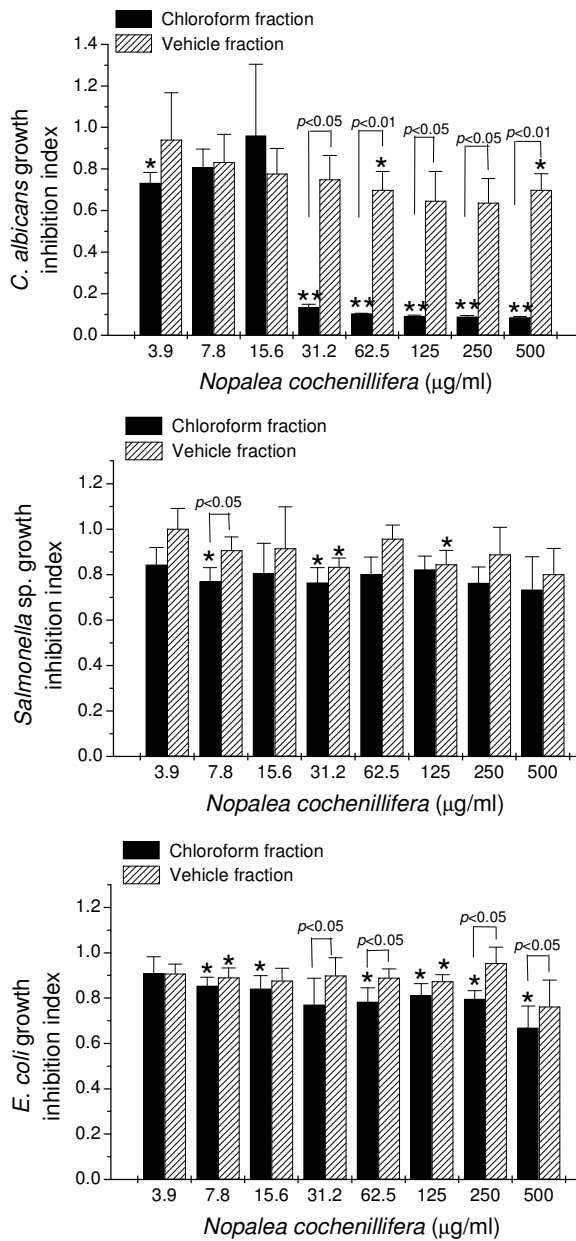
Fig. 5: Effect of dried Nopalea chloroformic fraction on *C. albicans*, *Salmonella enterica* and *E. coli* growth. *Candida albicans* was adjusted to 1 X 10³ yeasts mL⁻¹ in YM broth and bacteria were adjusted to 2x 10³ bacteria mL⁻¹ in ICC broth as explained in the text. Then bacteria and yeasts were cultured in the presence or absence of various concentrations of the Nopalea extracts or vehicle controls, for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which MTT was added to all wells and plates were incubated for 4 additional hours. At the end of the incubation period, extraction buffer was added to all wells and optical densities were read at 540 nm as detailed in the text. Data represent means ± SEM of growth inhibition indexes of triplicates from 3 independent experiments. **, P < 0.01; *, P < 0.05 as compared with untreated control
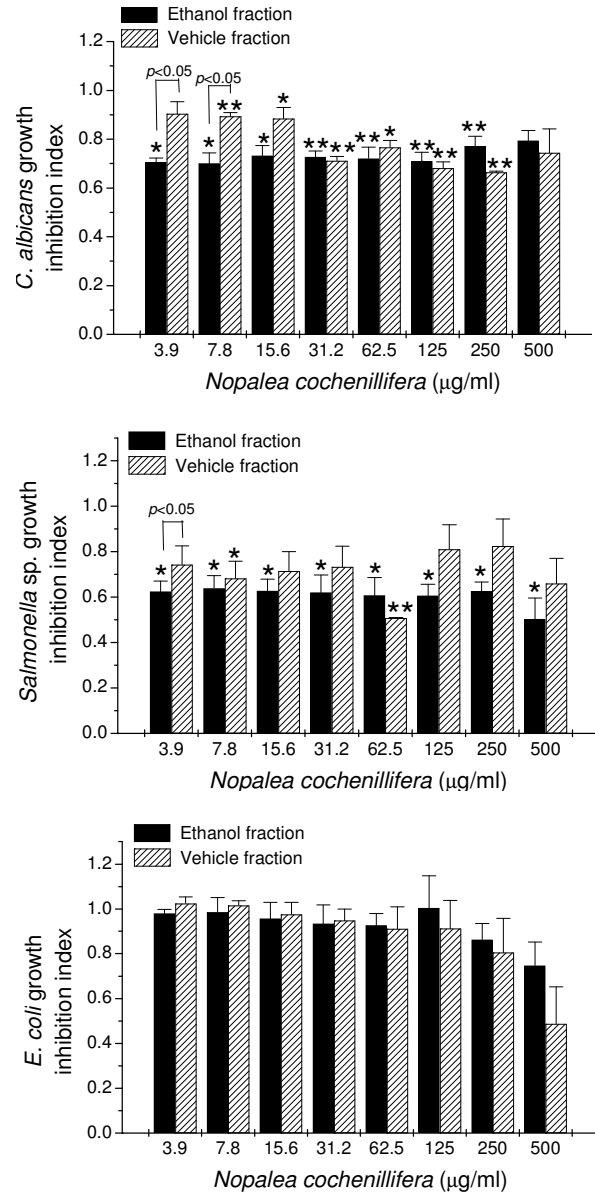
Fig. 6: Effect of dried Nopalea ethanolic fraction on *C. albicans*, *Salmonella enterica* and *E. coli* growth. *Candida albicans* was adjusted to 1 X 10³ yeasts mL⁻¹ in YM broth and bacteria were adjusted to 2x 10³ bacteria mL⁻¹ in ICC broth as explained in the text. Then bacteria and yeasts were cultured in the presence or absence of various concentrations of the Nopalea extracts or vehicle controls, for 24 h (yeasts) or 6 h (bacteria) at 37°C, after which MTT was added to all wells and plates were incubated for 4 additional hours. At the end of the incubation period, extraction buffer was added to all wells and optical densities were read at 540 nm as detailed in the text. Data represent means ± SEM of growth inhibition indexes of triplicates from 3 independent experiments. **, P < 0.01; *, P < 0.05 as compared with untreated control

but only the activity at concentrations 31.2, 62.5, 125 and 250 µg mL⁻¹ (89.5, 90.5, 91.1 and 91.5 percent growth inhibition respectively) were attributed to the effect of this fraction (P < 0.05) when comparing the

effect of the hexanic fraction versus the vehicle control (Fig. 4); any other observed inhibitory effect was due to the vehicle. This fraction also caused 24 to 32 percent *Salmonella enterica* var. thyphimurium growth inhibition at the concentrations tested, but only the

activity at concentrations 15.6, 31.2 and 62.5 µg mL^{-1} (30.9, 31.5 and 31.3 percent growth inhibition respectively) was attributed to the effect of the fraction (P < 0.05) when comparing the effect of the hexanic fraction versus the vehicle control (Fig. 4); any other inhibitory effect was due to the vehicle. In contrast, this fraction did not alter *E. coli* growth at all concentrations tested (Fig. 4).

Antimicrobial activity of dried *N. cochenillifera* chloroformic fraction: As observed in Fig. 5, Nopalea chloroformic fraction caused 4 to 91 percent *C. albicans* growth inhibition at the concentrations tested, but only the activity at concentrations 31.2, 62.5, 125, 250 and 500 µg mL^{-1} (86.4, 89.5, 90.8, 91 and 91.2 percent growth inhibition respectively) were attributed to the effect of this fraction (P < 0.05) when comparing the effect of the chloroformic fraction versus the vehicle control (Fig. 5); any other observed inhibitory effect was due to the vehicle. This fraction also caused 16 to 27 percent *Salmonella enterica* var. thyphimurium growth inhibition at the concentrations tested, but only the activity at 7.8 µg mL^{-1} (23 percent growth inhibition) was attributed to the effect of this fraction (P < 0.05) when comparing the effect of the chloroformic fraction versus the vehicle control (Fig. 5); any other inhibitory effect was due to the vehicle. This fraction was also associated with 9 to 33 percent *E. coli* growth inhibition at the concentrations tested, but only the activity at the concentrations 31.2, 62.5, 250 and 500 µg mL^{-1} (23, 22, 20 and 33 percent growth inhibition respectively) was attributed to the effect of the fraction (P < 0.05) when comparing the effect of the chloroformic fraction versus the vehicle control (Fig. 5); any other inhibitory effect was due to the vehicle.

Antimicrobial activity of dried *N. cochenillifera* ethanolic fraction: As observed in Fig. 6, Nopalea ethanolic caused 20 to 30 percent *C. albicans* growth inhibition at the concentrations tested, but only the activity at concentrations 3.9 and 7.8 µg mL^{-1} (29.4 and 30 percent growth inhibition respectively) were attributed to the effect of this fraction (P < 0.05) when comparing the effect of the ethanolic fraction versus the vehicle control (Fig. 6); any other observed inhibitory effect was due to the vehicle. This fraction also caused 36 to 50 percent *Salmonella enterica* var. thyphimurium growth inhibition at the concentrations tested, but only the activity at 3.9 µg mL^{-1} (37.6 percent growth inhibition) was attributed to the effect of this fraction (P < 0.05) when comparing the effect of the ethanolic fraction versus the vehicle control (Fig. 6); any other inhibitory effect was due to the vehicle. In contrast, this fraction did not alter *E. coli* growth at all concentrations tested (Fig. 6).

DISCUSSION

Medicinal plants have become part of complementary medicine worldwide, because of their potential health benefits. Plants have been used for centuries in the medical practices mostly in therapy of diverse pathologies[20]. Nopal is widely used in Mexico as a treatment for glucose control[21] and is known in the United States as prickly pear cactus. *N. cochenillifera* pads are rich in vitamin A and have been used for headaches, eye troubles and insomnia if consumed as a tea[21]. In addition, the split pads are emollient and used as poultices on rheumatism and baked for ulcers, gout and wounds. This plant is good for warts, kidney problems, measles and as a vermifuge for gastro-intestinal parasites, used sparingly. Other studies suggest that nopal stems and fruit might have anti-inflammatory, pain-relieving and stomach-protective effects[15]. However, important validation of its antimicrobial properties has not been fully determined. Some reports have shown inhibition of herpes simplex virus type 1 infection by *N. cochenillifera*[17] and antiviral activity of Opuntia streptacantha[21].

In our study, we showed that this cactus possesses antibiotic activity against *Candida albicans*, *Escherichia coli* and *Salmonella enterica* var. thyphimurium, which can cause persistent disease in human immunodeficiency virus type 1-infected people and other immunocompromised individuals[22,23]. We showed that hexanic and chloroformic fractions of dried *N. cochenillifera* were more effective than fresh pads in inhibiting *Candida albicans* growth; this may indicate a greater availability of the active compounds in the dried material (other benefits are the widespread use this material for traditional healers, it is easier to work with than fresh material and there are few problems associated with the large scale extraction) compared with the fresh pads and also gives indication of the non-polar nature of the involved compounds. The use of immunosuppressive drugs, the spread of AIDS and in general acquired immunosuppression, have resulted in an increasing occurrence of opportunistic systemic mycoses. Candidiasis of the esophagus and mouth by *Candida albicans*, is the most common infection observed in the immunosuppressed individual[24]. Because there are limited effective antifungal drugs currently available for the treatment of candidiasis, it is critical to discover and evaluate new sources of antifungal agents. *N. cochenillifera*-derived natural products may be of potential interest for novel antifungal agents. In addition, lower chloroformic and ethanolic concentrations of dried *N. cochenillifera* than those of fresh pads were needed to inhibit *Salmonella enterica* var. thyphimurium growth; this indicates that the putative bioactive compounds may become more available in the dried vegetal material and that both polar (proteins and sugars) and non-polar (mainly lipids) compounds are involved in this antibacterial activity. Additionally, only chloroformic fraction of both dried and fresh *N. cochenillifera* pads were capable to inhibit *E. coli* growth. In this case, non-polar

compounds from both dried and fresh pads are probably involved in the antimicrobial activity observed. Both *Salmonella enterica* var. thyphimurium and *E. coli* are well recognized not only to commonly cause gastrointestinal disorders, but also induce opportunistic infections in AIDS individuals[25,26]. Morbidity and mortality caused by bacterial infections importantly decreased upon the discovery and clinical use of antibacterial agents. However, nowadays public health faces the challenge of increasing mechanisms of antibiotic resistance of bacteria, because of the continuous and uncontrolled usage of antibacterial agents[27]. These mechanisms include decreased drug accumulation and permeability and modification of the antibiotic[28].

Plant antimicrobials are not currently used systemically because of their low activity, particularly against gram-negative bacteria. These compounds are usually classified as "antimicrobial" if they produce minimal inhibitory concentrations in the range of 100 to 1000 µg mL^{-1}, which are still higher than those of typical bacterial and fungal antibiotics (0.01 to 10 µg mL^{-1})[29]. However, in our study we have shown that some of Nopalea extracts possessed antimicrobial activity at concentrations as low as 7.8 mg mL^{-1} (Fig. 2, 5 and 6), which may be an indication of a potent antibiotic activity of such extracts and may be of physiological relevance[30]. Because of this, isolation and evaluation of novel antimicrobial agents from plants, particularly, *N. cochenillifera*, may lead to the discovery of natural antibiotics for which bacteria and other organisms are susceptible. In preliminary studies, we have observed that *N. cochenillifera* pads are rich in flavonoids and tannins (data not shown) which may be related to their biological activity. To our knowledge, this is the first report showing that Nopalea extracts affect bacteria and yeast growth. However, more investigations are needed to isolate and identify the molecular structure of the biologically active substances in *N. cochenillifera*. There are still a number of plant compounds that remain to be evaluated at the molecular, cellular and physiological levels for their potential to treat human diseases. Further studies are underway to evaluate the Nopalea´s extract and active compounds in vivo in a murine model of infection and to characterize the antimicrobial active compound (s).

ACKNOWLEDGMENT

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