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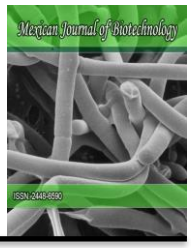

SHORT COMMUNICATION

Screening of anti-urease and antibacterial activities of root extract from *Cylindropuntia cholla*

Efecto anti-ureasa y actividad antibacteriana del extracto de raíz de *Cylindropuntia cholla*

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ABSTRACT

In this study, the root extract of *Cylindropuntia cholla* was analyzed as a possible source of substances with potential urease inhibitory and antibacterial activity. *C. cholla* cactus are abundant in the arid regions of Mexico and are traditionally used to treat kidney diseases, urinary tract infections, and in some cases, to combat the formation of kidney stones. Anti-urease action was evaluated from methanolic root extracts by initial velocity methods. We observed an IC_{50} of 2.04 mg/mL. Lineweaver-Burk pattern showed an uncompetitive inhibition. The antibacterial activity was evaluated over *Klebsiella pneumoniae* (ATCC™ 13883) and *Staphylococcus aureus* (ATCC™ 25923) and we only observed effects over *S. aureus* growth at 200 mg/mL of extract concentration.

Keywords: Cactacea, urea, kidney diseases, struvite and carbonate apatite

RESUMEN

En este estudio, el extracto de raíz de *Cylindropuntia cholla* se analizó como una posible fuente de sustancias con actividad potencialmente inhibidora de la ureasa, así como efecto antibacteriano. Los cactus *C. cholla* son abundantes en las regiones áridas de México y se utilizan tradicionalmente para tratar enfermedades renales, infecciones del tracto urinario y, en algunos casos, para combatir la formación de cálculos renales. La acción anti-ureasa se evaluó a partir de extractos metanólicos de raíz por métodos de velocidad inicial. Observamos una IC_{50} de 2.04 mg/mL. El análisis del diagrama de Lineweaver-Burk mostró una inhibición no competitiva. La actividad antibacteriana se evaluó sobre *Klebsiella pneumoniae* (ATCC™ 13883) y *Staphylococcus aureus* (ATCC™ 25923) y solo se observaron efectos sobre el crecimiento de éste último empleando una concentración de extracto de 200 mg/mL.

Palabras clave: Cactacea, urea, enfermedades renales, estruvita y carbonato apatita

1. INTRODUCTION

Urease is a nickel-dependent metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbamic acid. Carbamic acid is then spontaneously degraded into carbon dioxide and ammonia. Several species of bacteria and fungi have been shown to express the urease complex and the related ureolytic activity *in vitro* and *in vivo* in infections. The structure of nickel-dependent ureases obtained from microorganisms, fungi, and plants possess a striking sequence homology, and the active site is a highly conserved region (Fig. 1) (Mazzei *et al*., 2019). In some cases of bacterial urinary tract infections, a high activity of bacterial urease produces the accumulation of the metabolic product, ammonia, which has been implicated, at least partially, in the appearance of lesions of the mucosal tissue observed in the pathogenesis by these types of infections. One clinical complication during urinary tract infections with ureolytic bacteria is the formation of stones from the increase in the pH of the urine by ammonia ions reacting with ions of trivalent phosphate, calcium and carbonate. Subsequently, this reaction generates struvite and carbonate-apatite minerals in aqueous medium (Miano *et al*., 2007; Aslama *et al*., 2011). Notably, in 15% of clinical cases, this phenomenon is associated with infection by urease-producing microorganisms, predominantly species of the genera *Proteus*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* (Thomas & Tolley, 2008).

Different classes of compounds with inhibitory properties against urease have been previously investigated; among them are the phosphoramidates, boric acid hydroxamic acids, alkaloids and heavy metal ions. However, their effects are not well understood and most of these compounds are toxic or unstable, doing the search for urease inhibitors not trivial. Conceivably, one of the potential sources of new anti-urease/antimicrobial compounds is vegetal extracts, which is the present report's premise (Modolo *et al*., 2015; Zhou *et al*., 2017).

Cylindropuntia cholla is a cactacea belonging to the sub-family *Opuntioideae* and its distribution is concentrated mainly in arid zone of the Andes, Northwestern Mexico and

Southwestern USA. The phytochemical characterization of this plant has not been widely studied, although the root of the plant has different uses in ethnomedicine for the treatment of inflammation, influenza, stomach pain and urinary tract diseases, including complications with urinary stones (Balandrán-Quintana *et al*., 2018). In this work, it is postulated that roots are a potential source of anti-urease compounds. Therefore, this study aimed to evaluate the inhibition of urease activity by *C. cholla* root extracts and their antibacterial effect on some urease-producing bacteria commonly associated with urinary tract infections.

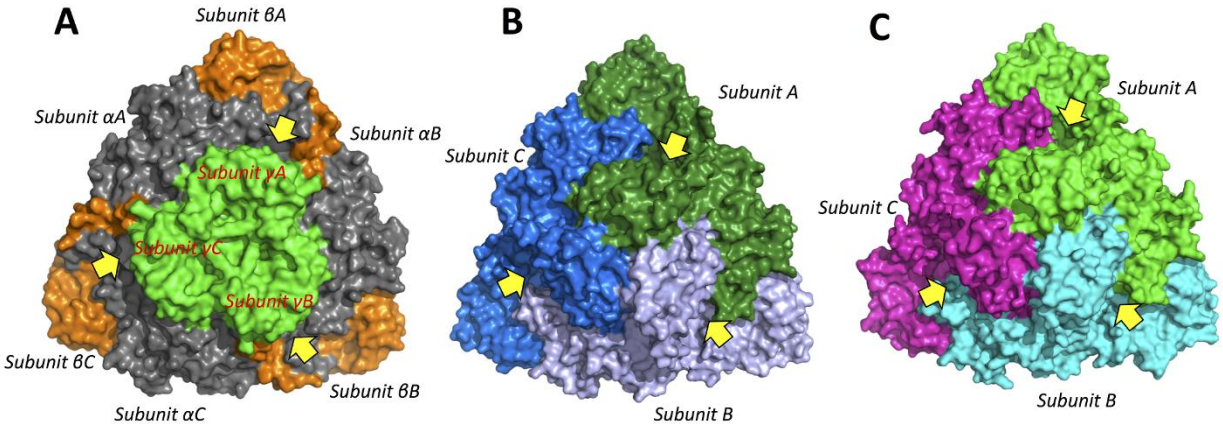

Fig. 1 Surface representation of structure of urease from different sources: (A) *Klebsiella aerogenes*, (B) *Canavalia ensiformis* y (C) *Aspergillus niger*, generated by authors with the UCSF-Chimera software, where conservation of structure and active site are shown.

2. MATERIALS AND METHODS

2.1. Plant extraction

Samples of *C. cholla* roots were collected in Southern Sonora State in Mexico (27° 53' N and 109° 55' W) and processed as previously reported (Núñez-Gastélum *et al*., 2015). Briefly, the roots were washed thoroughly with distilled water to remove soil residues, dried in the air to remove the excess of water and then stored in plastic bags at −80 °C (Thermo Scientific, Marietta, OH, EUA) for 24 h. After that, the samples were lyophilized (Labconco Freeze, Kansas City, MO, EUA) for 3 days at −48 °C and then powdered with a blender. Extracts were obtained by mixing 10 g of roots and 100 mL of methanol:water (J.T. Baker, Estado de Mexico, Mexico) mixture (80:20, v/v). The mixture was sonicated at 40 kHz for 30 min (Branson Ultrasonics Corp CPXH, Danbury, CT, USA), then centrifuged for 15 min at 1650 *g* (Eppendorf 5810R, Eppendorf Hamburg, Germany). The resulting supernatant was filtered and distilled at reduced pressure to remove methanol. The residue was recovered, frozen and lyophilized. The resulting extract was packed under vacuum and stored at −20 °C until later use.

2.2. Inhibition test of urease by *C. cholla* extract

Urease from seeds of *Citrullus vulgaris* was used to evaluate the anti-urease activity of methanolic *C. cholla* extract. The purified enzyme was prepared as we previously described (Díaz-Sánchez *et al*., 2016). For the inhibition test, 2 mM of urea concentration was added to the standard reaction buffer (0.5 mM MES, 0.016 mM phenol red, at pH 6.8) alone or mixed with different concentrations of the root extract (0−5 mg/mL). Reactions were initiated by adding 3 µL (50% of V_{max}, 1850 U/mg of protein) of the enzyme using a microplate reader FLUOstar Omega (BMG, Ortenberg, Germany). Lineweaver–Burk plots were performed and analyzed to determine the inhibition mechanism and pattern. All experiments were done in triplicates.

2.3. Antibacterial test

The antibacterial activity was evaluated by the extent of inhibitory halo using the disc diffusion method (Klančnik *et al*., 2010). Two bacterial strains, which are urease-positive and have been associated to urinary tract diseases, were used: *Klebsiella pneumoniae* (Gram-negative, ATCC™ 13883) and *Staphylococcus aureus* (Gram-positive, ATCC™ 25923). Briefly, 1 mL (1×10^5 CFU/mL) of each bacterial suspension was uniformly applied to a Petri dish containing sterile Müeller Hinton agar. Sterile filter paper disks (6 mm diameter, Whatman, GE Healthcare, Buckinghamshire, UK) were placed on each Petri dish followed by the addition of 10 µL of *C*. *cholla* extract at different concentrations (40, 100 and 200 mg/mL). Absolute ethanol and chloramphenicol (30 µg/mL, Sigma-Aldrich, Toluca, Mexico) were used as negative and positive controls, respectively. Assays were done by triplicate.

2.4. Statistical analysis

Data of inhibition halos were analyzed using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA) and were expressed as mean ± standard deviation (SD). Kinetics, curve fittings, and plots were prepared using Graph Pad Prism 5® (GraphPad Software, Inc., La Jolla, CA, USA).

3. RESULTS

3.1. Inhibition of urease by *C. cholla* extract

For urease isolated from *C. vulgaris,* the kinetics of the reaction of urea hydrolysis was determined. Figure 2 shows a hyperbola of the classical Michaelian type (Rozengurt *et al*., 1969). The enzyme presented a maximum velocity (V_{max}) of 0.74 ± 0.02 Abs/min and a Michaelis-Menten constant (K_m) of 6.54 ± 0.34 mM. The inhibition of *C. vulgaris* urease activity by *C. cholla* extract added to the reaction at a different range of

concentrations of 0 to 5 mg/mL is shown in Fig 3. The effect of the extract on urease inhibition was plotted as the initial maximal velocity (Abs/min) as a function of the extract concentration. As shown in Figure 2, when a constant amount of urease is tested versus increasing amounts of *C. cholla* extract, the maximal reaction rate (V_{max} as Abs/min) is diminished and even completely blocked at 4 mg/mL of the extract. The calculated IC_{50} for the extract is 2.04 mg/mL, which produced a reduction of both V_{max} and K_m (Fig 3 and 4A).

A kinetic inhibition assay was performed to determine the type of inhibition exerted by the extract. In this case, the substrate at a sub-saturation concentration (2 mM) was placed in contact with urease in the absence or presence of the extract (2 mg/mL) (Fig. 4). The Lineweaver–Burk plot was used to infer the inhibition mechanism. The pattern of two parallel lines with similar slope suggests an uncompetitive inhibition (Fig. 4B). The kinetics value of urease activity without extract had a K_M of 3.1 mM and V_{max} of 0.14 Abs/min; while those values when *C. cholla* extract was present in the reaction were 3.8 mM and 0.06 Abs/min, respectively.

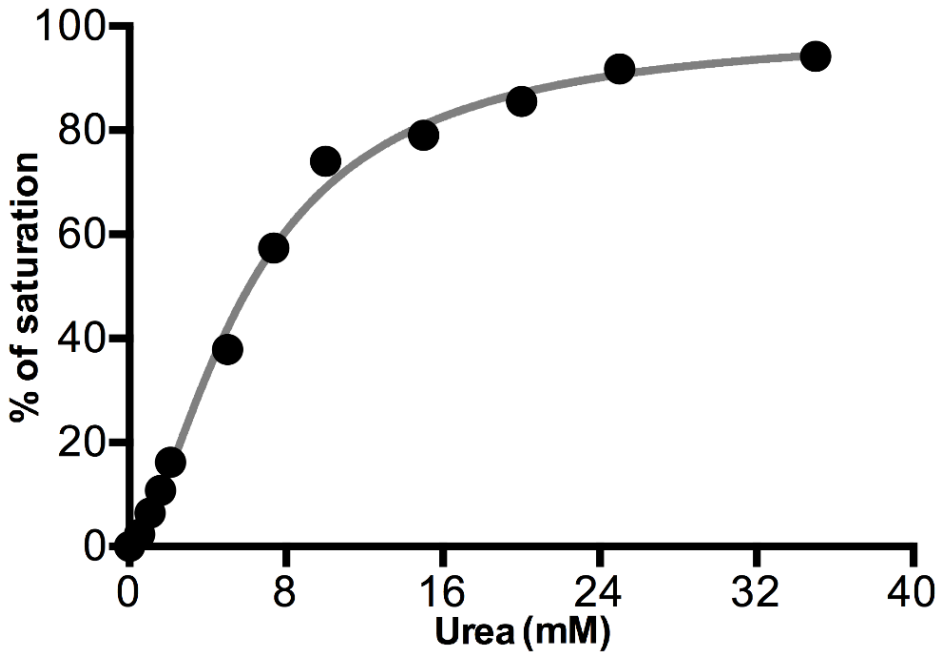

Fig. 2 Effect of substrate concentration on isolated urease activity (U/mL).

3.2. Antibacterial inhibition

An inhibitory effect of the microbial growth of *S. aureus* by *C. cholla* root extract was observed. When a concentration of 200 mg/mL is used, it resulted in an inhibition halos

of 9.3 ± 0.5 mm. Root extracts did not show any effect against Gram-negative bacteria *K*. *pneumoneae* proliferation.

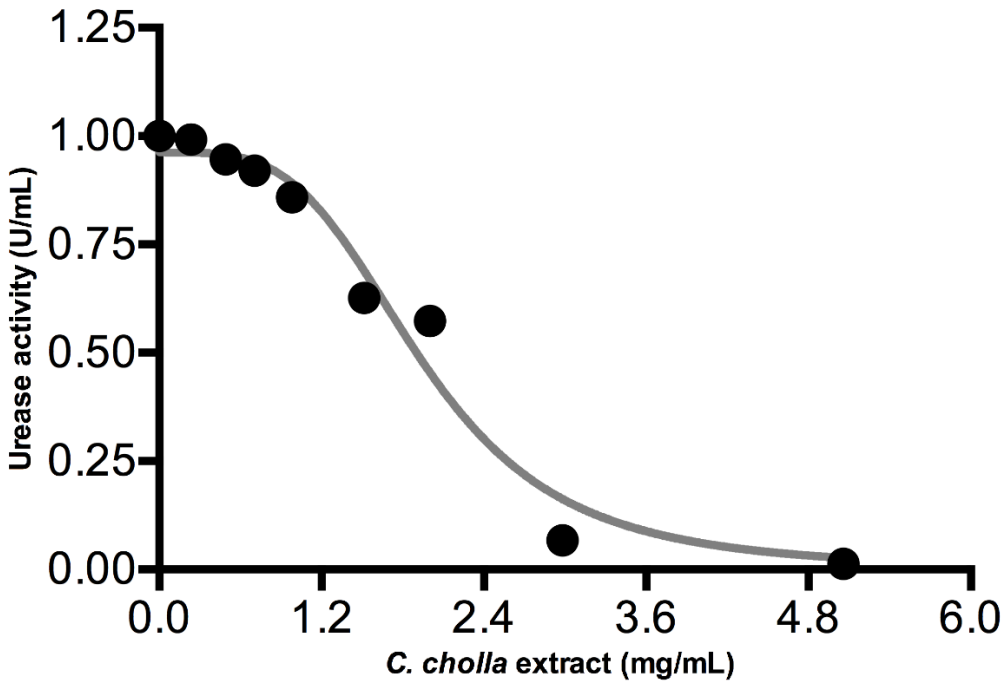

Fig. 3 Inhibition curve of the urease activity at increasing *C. cholla* extract concentrations keeping constant the concentration of urea (2 mM).

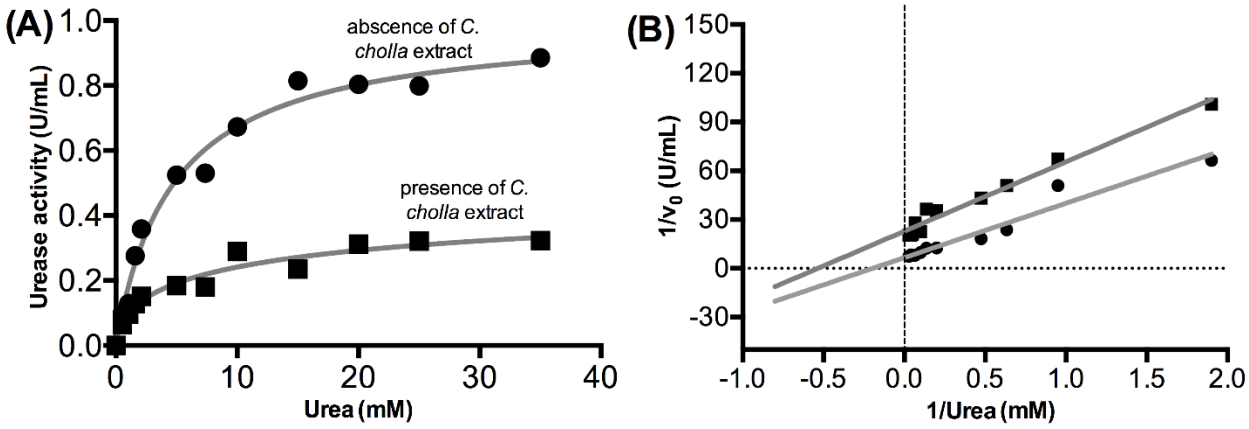

Fig. 4 (A) Effect of substrate concentration on urease activity; (B) Lineweaver–Burk plot.

4. DISCUSSION

Despite advances in bioinformatics, proteomics, transcriptomics, and gene expression, using secondary metabolites of natural products is still a viable route for identifying and designing new pharmaceutical products (Katz & Baltz, 2016). For example, there are no previous antibacterial studies in the case of *C. cholla* root. However, aqueous stem extract inhibited Gram-negative and Gram-positive bacteria species, including *K*. *pneumoniae* and *S*. *aureus* (McCleary & Walkington, 1964). In this report, we showed inhibition of proliferation for root extract only in the case of *S. aureus* but not *K. pneumonia.* However, it should be noted that the concentration of the extract used in the reference study was 2.5 times greater than that used in the present work. Other discrepancies with respect to our research may be due to several factors that influence the percentages of the active principles, such as the composition of the tissue analyzed, the time of harvest, environmental conditions in which the cactus grew, and method of extraction, among others (Katz & Baltz, 2016). Recently, Reyes-Becerril *et al*. (2022) reported the abundant presence of phenolic acids, flavonoids and phytosterols, mainly β-sitosterol and campesterol, in the aqueous extracts of *C. cholla*. These compounds have been widely related to an antibacterial effect (Coppo & Marchese, 2014; Luhata & Usuki, 2021). As a result of a hydrophilic extraction (methanol:water, 80% v/v), the extract in this study probably contained similar bioactive compounds. On the other hand, an antibacterial effect has been reported in root extracts of other cacti. Specifically, lipophilic compounds obtained from the root of *Opuntia ficus-indica* showed antibacterial activity, in ascending order, for *E. coli, B. cereus, P. aureaginosa* and *S. aureus* (Benramdane *et al*., 2022). Likewise, hexanic extracts from the *O. ficus-indica* flower inhibited the growth of *E. coli* and *S. aureus* (Ennouri *et al*., 2014). Furthermore, Sánchez *et al*. (2014) reported the antibacterial effect of methanolic extracts from the cladeodes of various cultivars of *O. ficus-indica* against *Clostridium perfringens, C. jejuni* and *V. cholerae*.

To the best of our knowledge, there are no previous studies on anti-urease activity of *C. cholla* extracts. While this is true, several reports evaluate natural plant-products as a source of urease inhibitors. Furthermore, the species *C*. *cholla* postulated in this study is a cost-effective option for its medicinal use, given its abundance in the Sonoran Desert region, where it is underutilized by local communities. In Mexico, *C*. *cholla* has been used empirically to treat medical conditions related to a high bacterial urease activity. In addition, our values of K_m and V_{max} found for *C*. *cholla* extract are similar to those reported for other sources (Modolo *et al*., 2015).

Uncompetitive inhibition, which occurs when an inhibitor binds to an enzyme-substrate complex, is typically observed in multisubstrate enzymes, which is not the case for urease. This observation can be explained because the inhibition site is potentially located in the flexible flap of the active site and not in the substrate binding subsite. Inhibition is produced because the inhibitor interaction is most likely with residues not involved with substrate binding, for example, with the critical cysteine involved in the flap dynamics, thus preventing the histidine that acts as a general acid from being correctly positioned at its proton transfer site. The decrease in V_{max} is associated with

the formation of the enzyme inhibitor-substrate inactive complex that decreases the formation of the enzyme-substrate complex and the appearance of the products. Meanwhile, K_m decreases due to the rapid disappearance of the enzyme-retained complex, displacing the equilibrium of the reaction to the right due to the increase of the enzyme's affinity for the substrate (Robinson, 2015; Diaz-Sanchez *et al*., 2016). However, some uncompetitive inhibitors can interact with a hydrolytic enzyme in a place normally occupied by the catalytic water (Todd & Hausinger, 2000). In the urease structure, each nickel atom is bind to a water molecule (Carter *et al*., 2009). This study provides insights to unravel the mechanism of phytopharmaceutical properties of *C. cholla*.

Besides polyphenolic compounds and phytosterols, the presence of azoles (4-Chloro-benzo-[1,2,5]thiadiazol-5-ol and 2-[1-pyrrolidino]ethyl]-5-phenyl-[1, 3,4]oxadiazole) has been reported in the root of *C*. *cholla* (Reyes-Becerril *et al*., 2022). Imidazoles, compounds of the azole family, are among the commercial urease inhibitors. Currently, there are limitations and concerns about the use of antiurease drugs, since they are known for their toxicity and low stability (Modolo *et al*., 2015). In addition to azoles, it is well known that some flavonoids can inhibit urease (Shin *et al*., 2005). *C. cholla* can be considered a novel source of possible compounds with an antiurease effect to replace current drugs.

In summary, the extract of the root of *C. cholla* presents substances with the capacity to inhibit urease activity. In general, data obtained in this report refer to uncompetitive inhibition. On the other hand, preliminary results do not show a remarkable antimicrobial activity by the extracts of *C. cholla*.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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