Antimicrobial effect of *Eysenhardtia polystachya* homemade extracts on bacteria causing urinary tract infections

Efecto antimicrobiano de extractos caseros de *Eysenhardtia polystachya* sobre bacterias causantes de infecciones del tracto urinario

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Abstract

Objective: To evaluate the *in vitro* antimicrobial effect of homemade preparations of *Eysenhardtia polystachya*, obtained by maceration or by infusion, on bacteria that cause urinary tract infections. Methodology: Microdilution assays were performed in 96-well plates where bacterial suspensions were co-incubated with different dilutions of homemade extracts, as well as a control for comparison using a commercial extract of the plant. Subsequently, the absorbance at 600 nm was measured to be considered as a direct measure of bacterial growth. For each culture, the values were normalized to growth percentage from the absorbance value obtained in the wells without extract. Contribution: The results show that the homemade extracts have a partial antimicrobial effect on the growth of the bacteria used in this work. The effect was the same between the extract by infusion and the extract by maceration against Gram negative bacteria. On the other hand, the extract by infusion showed a better effect than the extract by maceration on Gram positive bacteria. None of the homemade extracts achieved the antimicrobial effect of the commercial extract. This work corroborates the empirical knowledge of the use of homemade preparations of *Eysenhardtia polystachya* to treat urinary tract infections.

***Eysenhardtia polystachya* extract, Antimicrobial effect, Urinary tract, Bacterial infection**

Resumen

Objetivo: Evaluar el efecto antimicrobiano *in vitro* de preparaciones caseras de *Eysenhardtia polystachya*, obtenidas por maceración o por infusión, sobre bacterias causantes de infecciones del tracto urinario. Metodología: Se realizaron ensayos de microdilución en placas de 96 pozos donde se co-incubaron suspensiones bacterianas con diferentes diluciones de extractos caseros, así como un control para comparación usando un extracto comercial de la planta. Posteriormente, se midió la absorbancia a 600 nm para considerarla como una medida directa del crecimiento bacteriano. Los valores se normalizaron a porcentaje de crecimiento a partir del valor de absorbancia obtenido en los pozos sin extracto para cada cultivo probado. Contribución: Los resultados muestran que los extractos caseros tienen un efecto antimicrobiano parcial sobre el crecimiento de las bacterias utilizadas en este trabajo. El efecto fue el mismo entre el extracto por infusión y el extracto por maceración contra bacterias Gram negativas. Por otro lado, el extracto por infusión mostró tener mejor efecto que el extracto por maceración sobre bacterias Gram positivas. Ninguno de los extractos caseros logó el efecto antimicrobiano del extracto comercial. Este trabajo corrobora el conocimiento empírico del uso de preparaciones caseras de *Eysenhardtia polystachya* para tratar infecciones del tracto urinario.

Extracto de *Eysenhardtia polystachya*, Efecto antimicrobiano, Tracto urinario, Infección bacteriana

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Introduction

Mexico has a history of the use of ethnomedicine, whose origins go back to pre-Hispanic cultures where archaeological finds have demonstrated its influence and anthropological impact on Mexican culture. (Esquivel-García *et al*., 2018).

In Mexico, the National Commission for the Knowledge and Use of Biodiversity (Conabio, for its name in spanish), states that there are registered more than four thousand species of plants to which medicinal properties are attributed, representing almost 15% of the total flora of the country (CONABIO, s/f).

The World Health Organization (WHO) defined traditional medicine as "the set of knowledge, skills and practices based on the theories, beliefs and experiences of different cultures, whether explicable or not, used to maintain health and prevent diagnosing, ameliorating or treating physical and mental illnesses" (Organización Mundial de la Salud, 2013).

In recent years, the use of medicinal plants has increased for the treatment of various pathologies or as an adjuvant in medical treatments, an example of this is *Eysenhardtia polystachya*. This plant is commonly known as "blue wood" or "sweet wood", it belongs to the Leguminosae family. It is a deciduous tree or shrub 3 to 9 m tall with a diameter of 15 to 35 cm (Silva Guzmán *et al*., 2007). It has alternate, compound, pinnate crown and leaves, elliptic leaflets and aromatic resins.

The bark is rough and scaly with a dark color on the outside and reddish-brown on the inside (Silva Guzmán *et al.*, 2007). It presents inflorescences in pointed, lobed clusters with a white corolla. Its fruit is shown in a curved pod that houses a thin seed susceptible to water and is a hermaphrodite. It is distributed throughout Mexico, mainly in the states of Colima, Chiapas, Chihuahua, Coahuila, Mexico City, Durango, Guanajuato, Guerrero, Hidalgo, Jalisco, State of Mexico, Morelos, Oaxaca, Puebla, Querétaro, San Luis Potosí, Sinaloa, Sonora, Tamaulipas, Tlaxcala, Veracruz and Zacatecas. It has also been found in the southeastern United States (Gutierrez *et al*., 2015).

The recurrence of traditional medicine throughout Mexico is very common, since it is an essential part of Mexican culture as an alternative for the treatment of various pathologies or as an adjuvant in medical treatments. *Eysenhardtia polystachya* is used in traditional medicine to treat urolithiasis and other urinary diseases, such as urinary tract infections or the popularly called "urinary ailment". Commonly, the bark is boiled with water, obtaining a golden-colored liquid, which presents blue fluorescence and is administered orally (Pérez-Gutiérrez *et al*., 2002).

Urinary tract infections are a frequent problem in primary care and are among the most common infectious diseases worldwide. They are one of the leading causes of morbidity, only below respiratory infections (Tamadonfar *et al*., 2019). *Escherichia coli* is the main causative agent, being responsible for more than 90% of this type of infection. Other bacterial genera that also cause urinary tract infections include: *Klebsiella*, *Proteus* and *Staphylococcus* (Lee *et al*., 2018; Simões e Silva *et al*., 2020).

Until now, some biological properties of the ethanolic extracts of this plant have been reported, including antimicrobial activity. However, there are no studies that consider the dosage obtained in traditional preparations, which is how the population that uses traditional medicine consumes it. It is important to provide scientific evidence that supports the efficacy of the plant consumed in a traditional way, therefore, in this work the in vitro antimicrobial effect of the traditional preparation of *Eysenhardtia polystachya* against microorganisms that cause urinary tract infections is evaluated, in its preparations by infusion and maceration, taking as a reference the effect produced by a commercial extract of the same plant.

Methodology

Microorganisms and culture media

Three strains of Gram negative bacteria were used: *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 29906) and *Pseudomonas aeruginosa* (ATCC 27853) and two strains of Gram positive bacteria: *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213).

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All strains were propagated from a stock suspension stored at -80°C and streaked for colony isolation. For the propagation and maintenance of the bacteria, LB agar plates (1% peptone biotryptase, 1% yeast extract, 0.5% NaCl and 2% bacteriological agar) were used, where the strains were inoculated and incubated at 37ºC. For the determination of the minimum inhibitory concentration (MIC) by the broth microdilution method, Mueller-Hinton broth was used. To count the bacteria on the plate, peptone water (1% meat peptone and 0.5% NaCl) and tryptone glucose yeast extract agar (TGEA, 0.3% meat extract, 0.5% casein peptone, 0.1% glucose, extract of yeast 0.1% and bacteriological agar 2%).

Eysenhardtia polystachya extracts

The homemade extracts were made following the traditional preparation recommended by oral tradition. For this, the bark of the plant was purchased in a herbal medicine store and two extracts were prepared. For the first, a heaping tablespoon (15 g) of the bark was macerated in a liter of purified water overnight. For the second, 1 L of boiling purified water was added to 15 g of the bark and allowed to cool to room temperature. Both preparations were filtered separately and stored protected from light at 4°C until use.

To compare the results, a commercial extract that was purchased in the same herbal medicine store was used as a control (microdose of Palo azul, herbal extract, from the Tierra de vida brand). To use similar concentrations of the extracts (homemade and commercial) in the experiments, an aliquot was analyzed by UV-Vis spectrophotometry. From the spectrogram of the extracts, the commercial extract was adjusted by dilution until similar absorbance values were obtained. The highest concentration used in the experiments was that obtained in the undiluted traditional preparations.

Inoculum preparation

To obtain the inoculum from 0.5 MacFarland scale suspensions, 5 to 10 equal colonies were taken from the culture plate and diluted in 10 mL of peptone water.

Six decimal dilutions were then made using peptone water as diluent. 1 mL of the corresponding dilution was inoculated in duplicate in each box of medium and 18 to 20 mL of TGEA, melted and tempered in a water bath at 45 ± 1.0 ºC, were added. To homogenize, it was mixed with movements from right to left, clockwise and counterclockwise, and from back to front, on a smooth and horizontal surface until the complete incorporation of the inoculum was achieved in the medium, and they were left at rest until solidified.

One box without inoculum was included for each batch of medium and diluent prepared as a sterility control. The plates were incubated in an inverted position at 37 °C for 14 to 16 hours. To obtain an inoculum of 5 x 10^5 CFU/mL, the dilutions from which 50 colonies grew on the solid medium plate were considered. Based on this, the initial suspension was diluted in Mueller-Hinton broth for a concentration of 1 x 10^6 CFU/mL, so that when diluting the inoculum with an equal volume of extract, the final concentration would be 5 x10^5 CFU/mL.

Plate microdilution

The assay was performed in 96-well microplates using the serial dilution procedure as follows: for each extract, 100, 80, 60, 40, 20, and 0 μL of extract were placed in each well of the column, in duplicate. For those volumes less than 100 μL, the volume was completed with Mueller-Hinton broth. The same procedure was followed for the sterility control (100 μL broth + 100 μL of the extract dilution) and the negative control (100 μL broth + 100 μL water). Subsequently, 100 μL of the inoculum suspension were inoculated in all the columns except for the sterility controls, completing a final volume of 200 μL in the culture plate, thus adjusting the microbial population to 5x10^5 CFU/mL, and the extract/culture ratio at 1.0, 0.67, 0.43, 0.25 and 0.11. The plates were sealed and incubated at 37°C for a period of 18-24 hours. Bacterial growth was determined by measuring absorbance by UV-Vis spectrophotometry in a plate reader at a wavelength of 600 nm, to determine the lowest extract/culture ratio at which bacterial growth was inhibited.

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Statistic analysis

The absorbance values were normalized as a percentage of bacterial growth taking as reference the absorbance of the control without extract as 100%, for each condition (extract and bacteria) tested. All experiments were performed in duplicate and data from at least four independent experiments were used to report values as arithmetic mean ± standard deviation. Data were analyzed with a two-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism8 software for Windows. A *P* value less than or equal to 0.05 was considered significant.

Results

Concentration of homemade extracts

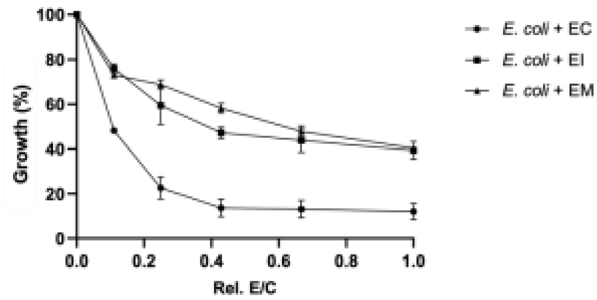
UV-Vis spectrograms of the two homemade extracts, obtained by maceration and by infusion, as well as the commercial extract used as control, were obtained. It was observed that the maximum absorbance peak was at the beginning of the visible range, at 400 nm of wavelength. And that both home preparations, infusion and maceration, reached similar concentrations (A_{400} ~ 0.130), while the commercial preparation was out of linearity.

Therefore, it was diluted until absorbance values below 1.0 were obtained. Thus, it was determined, in a 1:400 dilution, that the absorbance at 400 nm was 200 times greater for the commercial extract than for the homemade extracts. This suggests that home preparation methods do not allow optimal extraction of plant compounds.

Antimicrobial effect against Gram negative bacteria

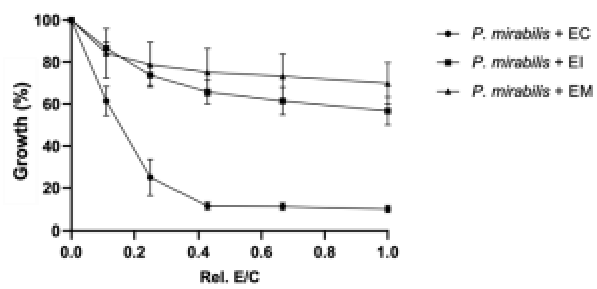
The antimicrobial effect of the homemade extracts obtained by infusion and by maceration on Gram negative bacteria that are commonly associated with urinary tract infections was tested. A commercial extract was used as a reference in each experiment. In Graph 1 it can be seen that the highest concentrations of home extracts manage to reduce almost 60% of the growth of the bacterial culture in vitro.

The statistics show that there is no significant difference in the effect of the homemade extracts among themselves (p = 0.4146), but there is a significant difference when any of the two homemade extracts is compared with the commercial extract in the same concentration ($p < 0.0010$). The commercial extract in a ratio of 0.11 extract/culture achieves the same effect as homemade preparations with a ratio of 1.0 extract/culture. The commercial extract in this last relationship manages to reduce bacterial growth by more than 80%.



Graph 1 Effect of *Eysenhardtia polystachia* extracts on the growth of E. coli. Rel. E/C: extract/culture ratio; EC: commercial extract; EI: extract by infusion; EM: extract by maceration
Source: Self Made

Graph 2 shows an effect similar to that observed for *E. coli* but in this case for *P. mirabilis*. Both homemade preparations manage to reduce almost 50% of bacterial growth, with no significant difference between them (p = 0.6192). There is a significant difference ($p < 0.0001$) when comparing any of the homemade extracts with the commercial extract, which manages to reduce 90% of bacterial growth from a ratio of 0.43 extract/culture, onwards.



Graph 2 Effect of *Eysenhardtia polystachia* extracts on the growth of P. mirabilis. Rel. E/C: extract/culture ratio; EC: commercial extract; EI: extract by infusion; EM: extract by maceration
Source: Self Made

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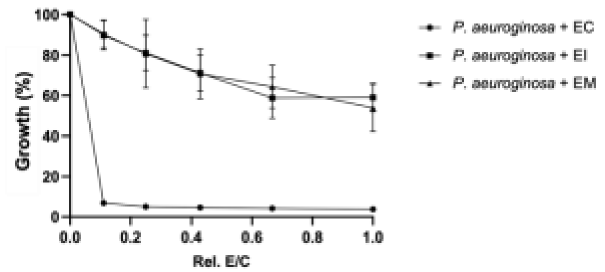
In the case of *P. aeuroginosa*, very low concentrations of the commercial extract managed to reduce around 95% of bacterial growth, contrasting with the effect that was observed with the homemade extracts where only a maximum reduction of 40% of bacterial growth was observed (Graph 3). There was no significant difference between both extracts (p = 0.9849), but there was between them and the commercial extract ($p < 0.0008$).

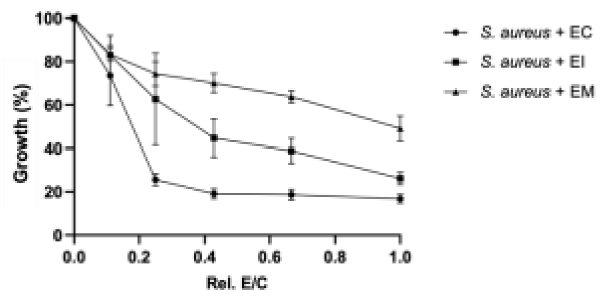


Graph 3 Effect of *Eysenhardtia polystachia* extracts on the growth of P. aeuroginosa. Rel. E/C: extract/culture ratio; EC: commercial extract; EI: extract by infusion; EM: extract by maceration
Source: Self Made

Antimicrobial effect against Gram positive bacteria

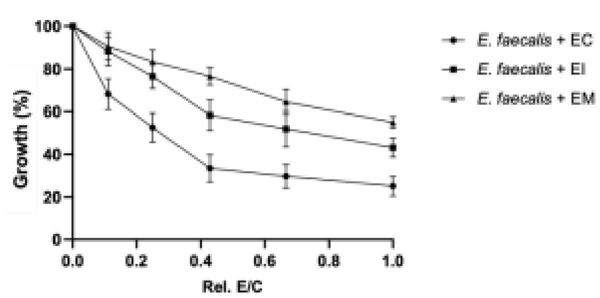
Unlike the almost homogeneous behavior shown by the home-made extracts by infusion and by maceration on the growth of Gram-negative bacteria, in the case of the Gram-positive bacteria used in this work, there were significant differences between the effect achieved by each of the preparations. homemade.

Graph 4 shows that the effect of the extract by infusion reduces the growth of *S. aureus* to levels very close to those achieved by the commercial extract (75% and 80%, respectively), both in the 1.0 extract/culture ratio; unlike the effect achieved by the extract by maceration that reduced growth by 50% in the extract/culture ratio of 1.0. The differences in the effect between the three extracts are statistically significant ($p < 0.0008$).



Graph 4 Effect of *Eysenhardtia polystachia* extracts on the growth of S. aureus. Rel. E/C: extract/culture ratio; EC: commercial extract; EI: extract by infusion; EM: extract by maceration
Source: Self Made

In Graph 5 it can be seen that the highest concentrations of the home extracts by infusion and by maceration manage to reduce almost 45% and 40%, respectively, of the growth of *E. faecalis*. The statistics show that there is a significant difference in the effect of the homemade extracts among themselves ($p < 0.0296$), and there is also a significant difference when comparing any of the two homemade extracts with the commercial extract at the same concentration ($0.0005 < p < 0.0120$). The commercial extract in a 0.25 extract/culture ratio achieves the same effect as home-made preparations by infusion with the highest extract/culture ratio. The commercial extract in this last relationship manages to reduce bacterial growth by more than 60%.



Graph 5 Effect of *Eysenhardtia polystachia* extracts on the growth of E. faecalis Rel. E/C: extract/culture ratio; EC: commercial extract; EI: extract by infusion; EM: extract by maceration
Source: Self Made

Discussion

Naturally, the microbiota only exists in the distal portion of the urethra, while the rest of the urinary tract remains sterile.

This microbiota is different in men and women. In the latter, due to the anatomical proximity of the urethral orifice to the perianal region, it is common to find *E. coli* that comes from the intestinal microbiota.

In the case of men, *P. mirabilis* is the bacterium most frequently found in the urethra. However, although it is common to find this type of bacteria in the urethra, an imbalance in their control usually precedes the development of urinary tract infections (Tamadonfar *et al*., 2019).

Among the plants most frequently used in traditional medicine to treat conditions related to the urinary tract, including infections, is *Eysenhardtia polystachya*. This is usually used in home extractions obtained by infusion or by maceration in drinking water. However, little has been studied about the antimicrobial capacity of this type of preparation to validate the effects attributed to this plant.

In this work we observed that home preparations do have an antimicrobial effect on both Gram negative and Gram positive bacteria, frequently associated with urinary tract infections, and that the effect they exert on the former is very similar between home preparations, regardless if they were obtained by infusion or by maceration. On the contrary, the homemade extract obtained by infusion has a greater effect than the one obtained by maceration on Gram positive bacteria. However, when comparing the effect of homemade preparations with a commercial extract of the same plant, it was observed that none of these was able to match the antimicrobial effect of the commercial extract. This may be due to the extraction method since both homemade preparations are aqueous and with very simple techniques. The extraction method of the commercial product is unknown, but it is likely that it comes from a larger amount of biomass and includes several processes, including purification.

According to the results of this work, it was verified that the home-made extracts of *Eysenhardtia polystachya* have a broad spectrum, since they act on Gram-positive and Gram-negative bacteria, which agrees with what was reported for methanolic extracts obtained from leaves of this plant (Rivas-Morales *et al.*, 2016).

Conclusions

Until now there are several studies on the biological activities of the alcoholic extracts of *Eysenhardtia polystachya*. However, little has been studied about the effects of the homemade extracts of this plant, which are frequently consumed in traditional medicine. In this work it was verified that the homemade extracts obtained by maceration and by infusion have an antimicrobial effect on Gram-positive and Gram-negative bacteria that cause urinary tract infections. Additionally, it was observed that the concentrations obtained in these homemade extracts are lower than those of the commercial extract, therefore, although they cannot replace pharmacological therapy, they can function as auxiliaries in the treatment of this type of infection.

Taken together, our results corroborate the empirical use given to the extracts by infusion or by maceration of this plant. Studies that include the administration of this type of infusion together with different doses of antibiotics can provide information on the synergistic or combined effect that these infusions can have when used as adjuncts in the treatment of urinary tract infections. All this with the purpose of reducing the doses of antibiotics necessary to achieve the therapeutic effect and thus reducing bacterial resistance to antibiotics.

Acknowledgments

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