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Antimicrobial potential of *Croton gossypiifolius* (Euphorbiaceae) latex on species associated with human infections

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The authors declare that they have no conflict of interest.

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Abstract: The empirical use of plants for medicinal purposes is an ancient practice. The *Croton* genus specifically is frequently utilized by indigenous communities in South America to treat a variety of infections. In this work, the potency of the latex coming from the *Croton gossypiifolius* (Euphorbiaceae) tree as antimicrobial agent was studied. Also known as "Dragon's blood", it was tested over microorganisms associated to infections in humans. The bacteria utilized were *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus, Pseudomona aeruginosa* and the fungi *Aspergillus niger*. The "Dragon's blood" was collected directly from the cortex of the trees by making V shaped cuts. It was then added to nutritious agar and saburoud plates, where the bacteria and fungi were grown separately for the optimum time and temperature of each species. An inhibition of the growth of *S. aureus* was observed. To quantify this inhibitory effect, *S. aureus* was cultivated in a Luria Bertani liquid medium adding different latex concentrations (0.6%, 1.25%, 1.9%, 2.5%, 3.3%) for 6 hours on each concentration. The growth was measured in Petri plates as colony-forming units per ml (CFU/ml). A dose dependent effect was observed. For 0.6% the mean growth was 900 CFU/ml, and the growth value decreased as the latex concentration increased until the smallest value of 10 CFU/ml was observed when the concentration was 3.0%. The latex did not cause any inhibition in the growth of the bacteria *E. coli, K. pneumoniae y P. aeruginosa*, nor the fungi *A. niger* but it did inhibit the growth of *S. aureus* and the effect was dose dependent

Keywords: *Croton gossypiifolius,* dragon's blood, *Staphylococcus aureus,* latex, natural antimicrobial.

Resumen: Potencial antimicrobiano del latex de *Croton gossypiifolius* (Euphorbiaceae) sobre especies asociadas a infecciones en humanos. El uso empírico de plantas con fines medicinales es una práctica ancestral, específicamente el género *Croton* es muy utilizado por comunidades indígenas de América del Sur para tratar diversas infecciones. En el presente trabajo se exploró el potencial como agente antimicrobiano del látex o savia del *Croton gossypiifolius* (Euphorbiaceae), también conocido como "sangre de drago" o "dragón", sobre microorganismos asociados a infecciones en seres humanos. Las bacterias utilizadas fueron *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus, Pseudomona aeruginosa* y el hongo *Aspergillus niger*. La "sangre de drago" fue colectada directamente de la corteza de los árboles haciendo cortes en "V" y se incorporó en las placas de agar nutritivo y saburoud, donde se cultivaron las bacterias y el hongo respectivamente, durante el tiempo y la temperatura óptimas para cada especie. Finalizado el ensayo se observó inhibición del crecimiento sólo de *S. aureus*. Para cuantificar el efecto inhibitorio se procedió a cultivar *S. aureus* en medio Luria Bertani líquido, adicionando diferentes concentraciones del latex (0,6; 1,25; 1,9; 2,5 y 3,3 %) con un tiempo de exposición de 6 horas, el crecimiento se midió en placas de Petri como unidades formadoras de colonia por unidad de volumen (ufc/mL). Se observó un efecto dosis dependiente, para 0,6% el crecimiento promedio fue de 900 ufc/mL, y decreció en la medida que se incrementó la concentración del latex, hasta un mínimo menor a 10 ufc/mL para la concentración máxima probada de 3,0 %: El latex no mostró efecto inhibitorio en las bacterias, *E. coli, K. pneumoniae y P. aeruginosa*, ni en el hongo *A. niger*, pero inhibió el crecimiento del of *S. aureus*, en forma dosis dependiente.

Palabras clave: *Croton gossypiifolius,* "sangre de drago", *Staphylococcus aureus,* latex, antimicrobiano natural.

INTRODUCTION

The use of natural compounds coming from plants for the treatment of diseases originates from previous millennia. It is understood that Neanderthal men that would occupy present day Iraq more than 60,000 years ago used plants for medical purposes (Stockwell, 1998). There has been documentation by early scientists and thinkers such as Hippocrates in ancient Greek medicine, Avicena in early Arab medicine, and Paracelsius in early Center-European culture. All of them were specialists in treating different diseases with medicinal plants (Gómez, 1990).

The *Croto*n genus is frequently utilized by South America's indigenous communities. It belongs to the Euphorbiaceae family. From these trees, the latex colloquially named "Dragon's blood" or "Drago's blood" is extracted to treat different diseases (Jones, 2003; Risco *et al*., 2005; INDECOCPI, 2007; Suárez *et al*., 2012). The first written documentation of its curative properties dates back to the XVII century when the explorer and naturalist Bernabe Cobo described the uses that the indigenous tribes from Mexico, Ecuador and Peru gave to this sap (Joyce, 1994). Among the best-known applications as a healing agent are due to its anti-inflammatory properties. Furthermore, it is may be used as an antiseptic and homostatic, in addition to other uses in traditional South American medicine, such as the treatment of gastrointestinal ulcers, colic, diarrhea and cancer (Sandoval *et al*., 2002; Suarez *et al.,* 2003; Tamariz *et al*., 2003; Suarez *et al*., 2006; Cevallos-Verdesoto *et al*., 2006); Gupta *et al.,* 2008; Suarez *et al*., 2009[A]).

Croton is including up to more than 1,300 species that include herbs, bushes, trees and unusually lianas widely distributed in tropical forests (Berry *et al.,* 2005). The chemistry and pharmacological composition of Venezuelan species have been described by Suárez *et al.* in different studies (Suarez *et al*., 2003; Suarez *et al*., 2009[B]; Suarez *et al*., 2012; Suarez *et al*., 2013). Specifically, the characterization observed from the latex of *Croton gossypiifolius* demonstrates that the majority of secondary metabolites that are present in the cortex of this tree correspond to diterpenes like the kaurenoic acid, grandiflorenic acid, ent-15β-Ecinamoil-16-kauren-19-oic acid, 7-desoxogeayin and quercitrin (Suarez *et al*., 2013).

The presence of these type of secondary metabolites such as the tapsin, proanthocyanidin SP-303 and other fenolic compounds such as the chlorequinic acid, coberins A and B, 1,3,5-trimetoxibenzene, and 2,4,6-trimetoxifenol, contribute to the "dragon's blood" medicinal and pharmacological properties described previously, as well as anti-microbial characteristics (Jones, 2003; Risco *et al*., 2005). In this regard, it has been shown that the bioactive compounds present in the *Croton lechleri* latex has anti-microbial effects against *Helicobacter pylori, Staphylococcus aureus*, *Pseudomona aeruginosa* and *Streptococcus mutans* (Tamariz *et al.,* 2003; Huapaya *et al.,* 2003; Risco *et al*., 2005; Cayo & Barrera, 2014; Avilés *et al.,* 2018) and against the herpes virus, hepatitis, and influenza (Gilbrty *et al.,* 1993; Sidwell *et al.*, 1994). Nevertheless, it has shown little to no activity against bacteria such as *Escherichia coli* and *Bacillus subtilis,* as well as against the human cytomegalovirus (Risco *et al*., 2005).

Currently there is a growing need to implement the use of this type of herbal product for the treatment of different types of infections and to meet the health needs of the population, especially given the problems caused by bacterial resistance (Chinin & Cisneros, 2018). Nosocomial infections are a public health issue of great economic and social importance. The association of these infections with the increase in microbial resistance represent a challenge for health institutions and medical personnel due to high rates of morbidity and mortality, the increase in hospitalization days, as well as the costs of care (Cervantes-García *et al*., 2014). Therefore, it is important to explore natural alternatives for the control of this type of microorganisms.

In regards to the above information and of the arsenal of secondary metabolites present in the latex of these plants, an exploratory study was performed to determine the antimicrobial capacity of the *Croton gossypiifolius* latex against strains associated with common infections in humans such as *Klebsiella pneumoniae, Pseudomona aeruginosa, Escherichia coli* and *Staphylococcus aureus*, and the fungi *Aspergillus niger.* These microbial species were chosen, particularly because many are associated with nosocomial infections.

MATERIALS AND METHODS

Biologic material

The "dragon's blood" latex was collected from *C. gossypiifolius* trees located in the forest zone of Cerro el Volcán (10°25'27.10" N, 66°51'35.81" O) at 1164 meters above sea level, which belongs to the Chacao municipality (Miranda- Venezuela). The process for collecting latex or bleeding from the tree was done according to what was traditionally established, with oblique incisions or V-cuts in the bark of the tree (Risco *et al.,* 2005). The exudate was collected in sterile plastic containers, which were well sealed to prevent solidification of the latex until later use.

Microorganisms

We worked with three certified strains of bacteria *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, donated by the National Hygiene Institute "Rafael Rangel". Clinical isolates of *Pseudomona aeruginosa* and *Aspergillus niger* were also used, donated by the Microbiology laboratory from the Microbiology Department of the Health Sciences Faculty, Carabobo University. The strains were stored under freezing temperatures until used in the experiments at the Biomedical Research Institute facilities "Dr. Francisco Javier Triana Alonso" of the Carabobo University in Maracay, Venezuela.

Antibacterial activity

Classic microbiology techniques were used to determine whether or not the extract affected the growth of the selected bacteria. The study was conducted in two stages; the first one was qualitative, in which two Petri dishes with nutritive agar were prepared per bacteria, one impregnated with the "Dragon´s blood" latex and the other one without it in order to compare the growth of bacteria, which were cultured with the swab technique. The second quantitative stage in which the

bacteria susceptible to the latex were selected and cultured in a liquid medium at different concentrations thereof for a period of time to subsequently sow them on agar plates and quantify the number of viable cells; this was done in order to determine if the effect depended on the concentration used.

For the development of the qualitative stage a pre-inoculum of the bacteria was performed in fiolas with 20 mL of liquid Luria-Bertani medium incubated at 37ºC under continuous agitation to 150 rpm during 24 h inside an incubator Labnet model 211DS. After that time the turbidity was determined at 600 nm in a spectrophotometer Beckman DU 650, and the bacterial solution was adjusted to 0.5 absorbance units per mL. In parallel, four Petri dishes with nutritive agar were prepared for each bacteria, two impregnated with Dragon's blood and two without it to serve as control. Sowing was performed using the diffusion technique with sterile swabs, which were impregnated with the different bacterial solutions. The dishes were incubated at 37 °C with an inverted position during 24 – 48 h.

In the quantitative stage, as in the previous case, a pre-inoculum of the bacteria was made and a volume equivalent to 0.5 absorbance units was taken which was incubated in flasks with liquid LB medium in the presence of the latex with different concentrations (0%; 0.6%; 1.25%; 1.9%; 2.5%; and 3.3 %) in a final volume of 20mL. It was incubated during a 6h period at 37 °C under continuous agitation at 150 rpm. After the incubation time, serial dilutions were made which were seeded in the Petri dishes with nutritive agar by the immersion technique and incubated for 24h at 37 ºC. Accounting plaques were considered to be those that ranged from 10-299 colonies. This procedure was performed in duplicate for each concentration tested.

Antifungal activity

To determine if the latex interferes in the growth of the *Aspergillus niger* strain, a procedure similar to the one developed by Falco *et al.* (2011), with *Aspergillus oryzae* was done. Unlike the antibacterial procedure, saboraud medium was used in the Petri dish. A 0.5 cm portion of the mycelium of the fungus was seeded in the center of the Petri dish with medium plus latex. The plates were incubated at 30 °C for 7 to 10 days and growth was observed and compared with a sample of mycelium grown under the same conditions, without the presence of the latex.

Statistical analysis

In view of the fact that the study developed was of the exploratory type, only a descriptive statistic of averages and standard deviation was applied to the results of plate counts.

RESULTS AND DISCUSSION

Antibacterial activity

When comparing the growth of the bacteria in the dishes with solid LB medium impregnated with the *C. gossypiifolius*

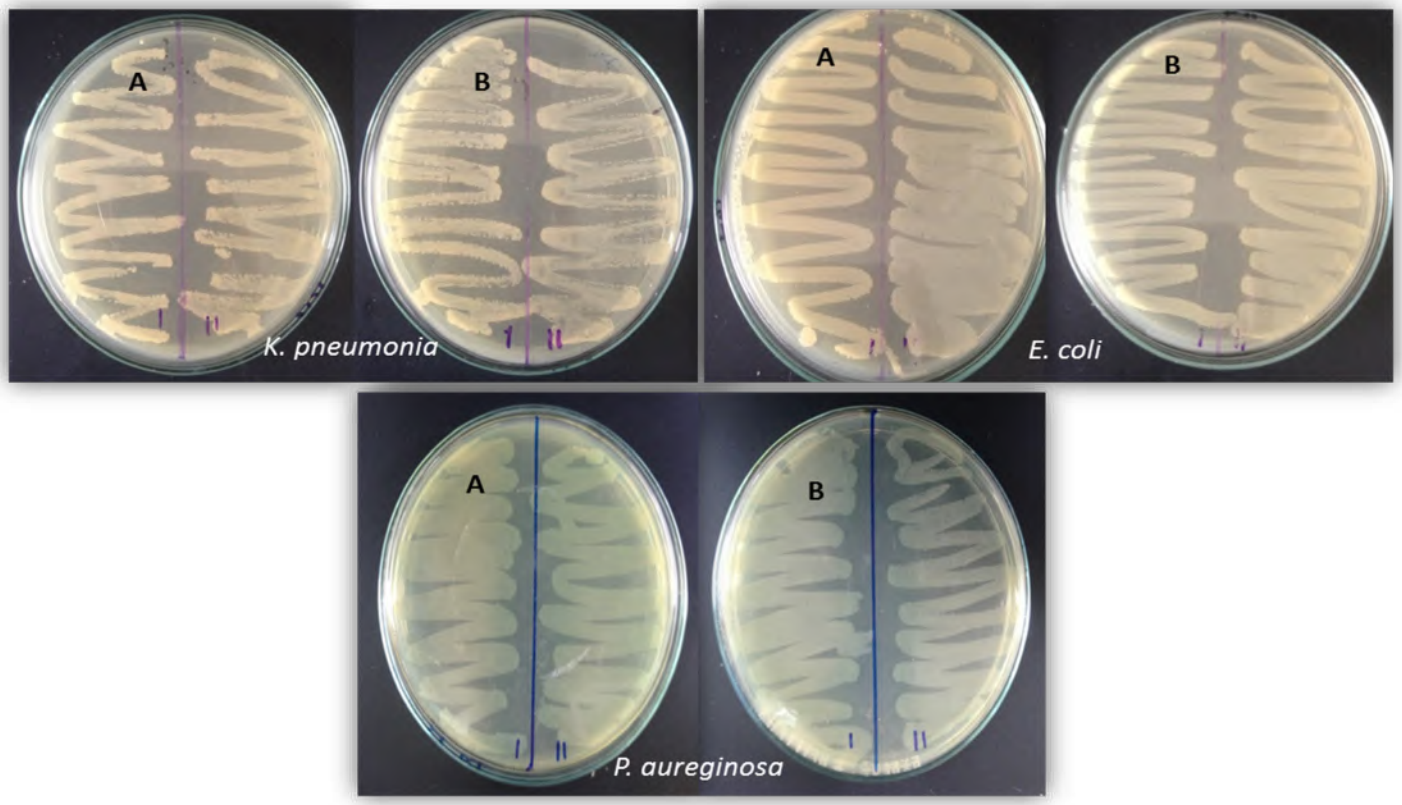

Figure 1. Plates cultivaded with bacteria: A) 100% latex concentration, B) control plate without latex.

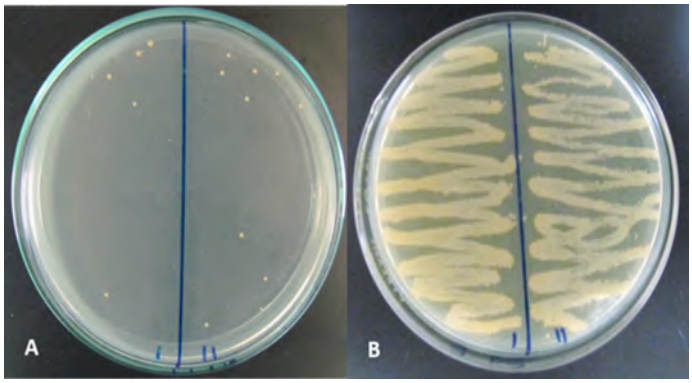

Figure 2. Plates cultivated with S. *aureus*: A) 100% latex concentration, B) latex control board.

latex with its respective control (medium without latex), it was observed that it did not cause growth inhibition of *E. coli*, *K. pneumoniae* y *P. aeruginosa* (Figure 1). Nor did it cause inhibition on the growth of *A. niger* (Figure 3). In the case of *S. aureus,* a significant reduction in growth was observed in the presence of latex (Figure 2).

This result coincides with Chininin and Cisneros (2018), findings, whom used 100% *Croton lechleri* latex with the disk diffusion test and achieved a 54.75% inhibition of the in vitro growth of *S. aureus* ATCC 25923 strain.

Based on these results, *S. aureus* was grown in LB medium with different concentrations of the latex for a period of 6 hours and then cultured in agar in order to quantify the number of viable bacteria (cfu / mL). An evident dependence on the concentration used for the inhibitory effect was observed. The highest bacterial counts were reached when the lowest (0.6%) latex concentration was used; while at 3.3% the growth was minimal (Figure 4). With these data, a linear regression analysis was carried out to determine the magnitude of this effect of the concentration on the number of viable bacteria. A negative slope of -430 was obtained ($r^2 = 0.94$), which implies that there was a reduction of 430 CFU / mL per unit of added concentration of the latex in the culture medium. As seen in the figure there is a strong tendency to inhibition, showing a concentration-dependent behavior.

All these results are very important due to the relevance of this bacterium in daily medical practice. Since its discovery in 1880 by the doctor Alexander Ogston, *S. aureus* is considered a pathogen with great potential to cause multiple infections in humans and animals, which affect the skin and soft tissues and can sometimes be fatal (Cervantes-García *et al*., 2014; Chinin & Cisneros, 2018). It is one of the most prevalently isolated bacteria in communities as well as nosocomial infections at the skin level, central nervous system, respiratory system, urinary tract; capable of producing localized abscesses, osteomyelitis, endocarditis, food poisoning and septicemia (Dorante *et al.,* 2013). One of the main issues associated with treating these infections is antibiotic resistance. The first oxacillin resistant *S. aureus* strains appeared in 1960, shortly after the introduction of this antimicrobial in clinical practice (Gómez-Gamboa *et al*., 2016) and until now, resistance mechanisms continue to evolve at the same rate as new drugs are modified or created.

Consequently, the findings of this work on the effectiveness of *C. gossypiifolius* latex to inhibit the growth of this bacterium profile this natural product as a therapeutic alternative to meet the health needs of the population. It is necessary to deepen the investigation performing standard sensitivity tests on these bacteria, including strains from clinical isolates.

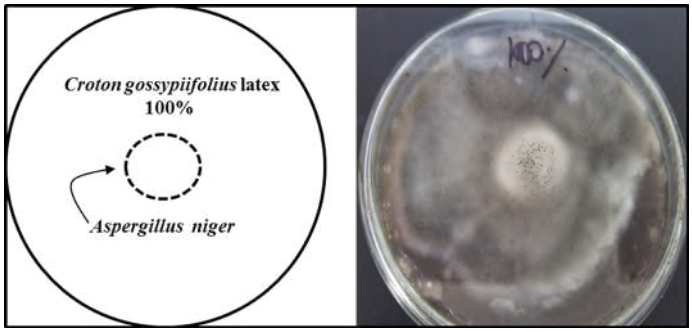

Figure 3. Sabaourad agar plate impregnated with 100% latex grown with *Aspergillus niger* for 10d/30 ° C.

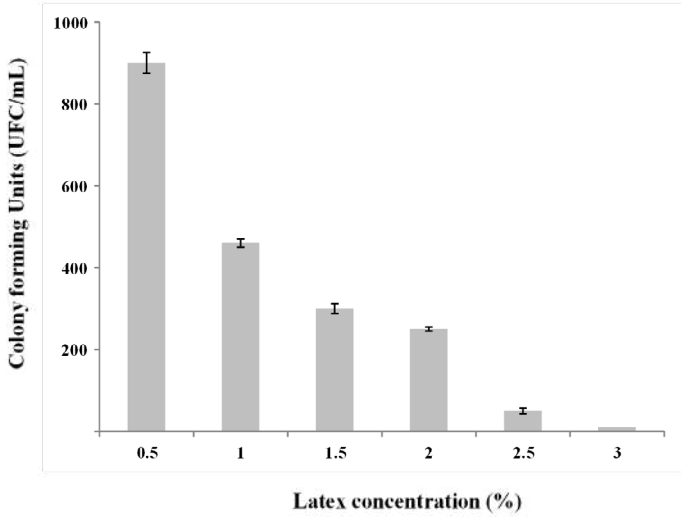

Figure 4. Variation of the viable bacteria count of the *Staphylococcus aureus* ATCC 25923 strain depending on the latex concertation.

CONCLUSION

The latex of *Croton gossypiifolius* did not cause any inhibition in the growth of the bacteria *E. coli, K. pneumoniae y P. aeruginosa*, nor the fungi *A. niger* but it did inhibit the growth of *S. aureus* and the effect was dose dependent. Consequently, it is important on deepen the study focused on *S. Aureus* infectious agent.

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