

***In vitro* effectiveness of an aqueous extract of neem (*Azadirachta indica* A. Juss) leaves on bacteria causing healthcare associated infection in Valledupar**

Efectividad *in vitro* del extracto de hojas de neem (*Azadirachta indica* A. Juss) sobre bacterias causantes de infecciones asociadas a la atención en sanitaria en Valledupar

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Abstract

Background: Globally, the need to address strategies for preventing infections associated with health care has increased worldwide. In the city of Valledupar, Colombia, reports of bacteria resistant to chemical or enzymatic biocides in hospital environments and surfaces are increasingly frequent, evidencing the importance of conducting studies aimed at identifying alternative active ingredients for disinfectant products.

Objective: Evaluate the *in vitro* effectiveness of Neem leaves extract over bacteria strains isolated from different areas and surfaces of a health institution in Valledupar, compared to disinfectants for hospital use, an enzymatic detergent, and a commercial chemical disinfectant.

Methods: Biocidal activities on bacteria isolated from hospital surfaces, such as *Acinetobacter baumannii*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus sp.*, and *Streptomonas maltophilia* were analyzed. The Neem leaves extract was evaluated at concentrations of 3, 4, and 5 % for

each bacterium during 15-minute contact time, incubated at 37 °C for 18 hours. We compared two antimicrobial chemicals, a disinfectant (based on formaldehyde, cetrimide, and glutaraldehyde), and an enzymatic detergent (based on protease, lipase, and amylase).

Results: The aqueous Neem extract did not show significant differences with the other treatments with 99.48 to 100% inhibition against bacteria of the species *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Staphylococcus aureus*, and *Micrococcus sp.*; *Bacillus subtilis*, and *Stenotrophomonas maltophilia* strains were the most resistant strains inhibited by enzymatic detergent and disinfectant, respectively. None of the products evaluated were effective against all *in vitro* strains.

Conclusions: These data show Neem's bacteriostatic properties, its potential in in-hospital products and the need to combine different active ingredients in a disinfection plan.

Keywords: antimicrobial, Neem Tree, cross infection *Acinetobacter baumannii*, *Staphylococcus epidermidis*

Resumen

Antecedentes: A nivel mundial ha aumentado la necesidad de abordar estrategias para la prevención de infecciones asociadas a la atención de salud. En la ciudad de Valledupar, Colombia, cada vez son más frecuentes los reportes de bacterias resistentes a biocidas químicos o enzimáticos, en ambientes y superficies hospitalarias, lo cual evidencia la importancia de realizar estudios orientados a la identificación de principios activos alternativos para productos desinfectantes.

Objetivo: Evaluar la efectividad *in vitro* del extracto de hojas de Neem en cepas de bacterias aisladas en diferentes áreas y superficies de una institución de salud en Valledupar, en comparación con desinfectantes de uso hospitalario, un detergente enzimático y un desinfectante químico comercial.

Métodos: Se analizó la actividad biocida sobre las bacterias aisladas de superficies hospitalarias *Acinetobacter baumannii*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus sp.* y *Streptomonas maltophilia*. El extracto de hojas de Neem se evaluó a concentraciones de 3, 4 y 5% para cada bacteria durante un tiempo de contacto de 15 minutos, incubados a 37 ° C durante 18 horas. Se comparó dos productos químicos antimicrobianos, un desinfectante (a base de formaldehído, cetrimide y glutaraldehído), y un detergente enzimático (a base de Proteasa, Lipasa y amilasa).

Resultados: El extracto acuoso de Neem no presentó diferencias significativas con los demás tratamientos con inhibición del 99,48 al 100%, sobre bacterias de las especies *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Staphylococcus aureus* y *Micrococcus sp.*; las cepas *Bacillus subtilis* y *Stenotrophomonas maltophilia* fueron las más resistentes a ser inhibidas por el detergente enzimático y el desinfectante, respectivamente. Ninguno de los productos evaluados fue efectivo contra todas las cepas *in vitro*.

Conclusiones: Estos datos evidencian las propiedades bacteriostáticas del Neem, su potencial en productos de uso intrahospitalario y la necesidad de combinar diferentes principios activos en un plan de desinfección.

Palabras claves: Antibacteriano, Infección hospitalaria, Neem, *Acinetobacter baumannii*, *Staphylococcus epidermidis*

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Introduction

According to the Pan American Health Organization (PAHO) and World Health Organization (WHO), approximately 1.4 million people acquire a Health care Associated Infection (HAIs), affecting 1 of 20 hospitalized patients, affecting about 4.1 million patients, and approximately 37,000 patients die each year (1). Furthermore, the *Acinetobacter baumannii* complex is the most frequently isolated pathogen with 80% of all clinical isolates and has a potential resistance of 3.1% to carbapenems (2).

These cases have become the most frequent events in health care, affecting several patients annually in many countries of the world, generating an impact on the quality of life of patients. The economic expenses attributable to them estimated for the US a cost of care of 28 and 33 billion dollars a year (3).

In Colombia, the cost of care for HAIs is COP 1,190,879 per person, in antibiotic treatment (41% of the total value) and laboratory analysis (13.5%) (4). Specifically, there are records of the presence in the hospital facilities of bacteria resistant to bactericides in Valledupar. 50% of *S. aureus* strains showed resistance to methicillin, up to 14% were isolated from the emergency and surgery areas (5).

The reason why the Ministry of Health and Social Protection of Colombia in 2018 created the prevention, surveillance, and control Program of HAIs and antimicrobial resistance, was to provide elements to strengthen the prevention and control of HAIs. Considering among other measures, the appropriate cleaning and disinfection practices, including the disinfectants selection for hospital use (1).

Consequently, using inside the disinfectant's composition natural ingredients with high biocidal power has been increasingly highlighted. Among those are extracts and essential oils of aromatic plants such as *Cymbopogon citratus*, *Lippia alba* (Mill), *Eugenia caryophyllata* (Thunb), Aloe vera (L.), *Azadirachta indica* (A. Juss), and others (6,7). The last one is a meliaceae family plant, currently used in the pharmacopeia, cosmetics, and phytosanitary products. This plant has active natural substances in leaves and seeds against multiple pathogens, including bacteria, fungi, and viruses (8). Its extract has been demonstrated to contain relevant properties at the health level (9,10), with antibacterial, anti-inflammatory, antiviral, hypoglycemic, and antiulcer activity, among others (11). Furthermore, experimental studies indicate that the ethanol extract of leaves has antibacterial activity *in vitro* against *Staphylococcus aureus* (12). Specific structural components within the Neem extract, such as deacetylgedunin (DCG), presents a high level of coupling with the PLpro protein of SARS-CoV-2, which allows its inhibition (13). Balakrishna et al. (7) developed a hand sanitizer gel containing *Azadirachta indica*, *Ocimum sanctum* and *Citrus limon* extracts, with an inhibitory effect on bacteria related to HAIs *Escherichia coli* and *Staphylococcus aureus*.

This research raised the evaluation of the bacteriostatic effect of the extract of the leaves and seeds of the Neem tree against microorganisms' strains isolated from surfaces in procedure rooms, neonatal intermediate care unit, adult intermediate care unit, and surgery of a hospital institution in Valledupar.

Materials and methods

Location

The study was carried out in the laboratory of the University of Santander, in Valledupar, Colombia.

Microorganisms

The bacteria associated with IAAS, *Acinetobacter baumannii*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus* sp., and *Streptomona malthophila*, were isolated by the method of a swab from different surfaces such as the surgical procedure room and the intensive care unit (ICU) (adult and neonates ICUs) from a hospital in Valledupar township. The bacteria grown at 37 ° C amid nutrient agar and Muller Hilton cultures. The strains were identified by the API 20E method for enterobacteria and API 20NE for other gram-negative bacilli. 0.85% saline suspensions were prepared on the Macfarland 0.5 scale to conduct the tests.

Extract from *A. indica* (Neem)

The aqueous Neem extract was prepared according to the methodology described by Barrabí and Garcia (14), where the plant material was manually collected in sterile plastic bags with airtight closure. Leaves and seeds were obtained from 10-year-old Neem trees located in the Centro Biotecnológico del Caribe (CBC)-SENA in Valledupar, Colombia, (169 MAMSL and an average temperature of 28 °C). The material was washed with distilled water, allowed to dry at 28 °C. Afterward, 5 g was weighed on a precision analytical balance, and 200 ml of distilled water was added in a flask and boiled for 25 minutes. Finally, the mixture was filtered on filter paper in a Büchner funnel, and a final volume of 100 ml of 5% Neem extract was obtained. Three concentrations were prepared from the stock solution: 3, 4, and 5%.

Chemical products

This research evaluated the products frequently used to disinfect environments, instruments, and surfaces from the hospital. The first product corresponds to a pre-disinfectant enzymatic detergent (protease, lipase, and amylase; liquid presentation, pH 8.9) used for instrument cleaning. The second product is a microbial disinfectant based on formaldehyde, cetrimide, and glutaraldehyde (400, 600, and 1000 ppm).

***In vitro* Effectiveness Comparison**

We prepared 9 ml of the enzymatic detergent, the disinfectant, and Neem extract at 3, 4, and 5% concentrations. Afterward, 1 ml of the microorganisms at 0.5 Macfarland scale was added, and let them mix during 15 minutes of contact. Finally, we inoculated the mix in-depth in plates containing 1 ml of the Muller Hinton agar suspension and incubated at 37 °C. Each strain was assessed in triplicate. By calculating the percentage of dead cells: $[1 - (\text{Mean CFU irrigant} / \text{Mean CFU}_{\text{initial bacterial number}})] \times 100\%$. Where “mean CFU irrigant” refers to the measurement of the colony-forming units resulting from exposure with the antimicrobial or irrigant substances to be evaluated and “Mean CFU_{initial bacterial number}”, the measurement of the initial bacterial colony-forming units, before being in contact with antimicrobial substances (15). Likewise, the enzymatic detergent and disinfectant were used in concentrations of 3% and 100%, respectively, assuming a high organic matter concentration. The density of application solutions is assumed to be 1.

Statistical analysis

According to Tukey, the median of the results was compared by analysis of variance followed by a 5% post hoc test using the statistical software SPSS version 20.

Results

In this research, when reviewing the results of the different treatments to which all the strains of bacteria evaluated were subjected, it was evidenced: 1) there was significant differences between extract of Neem concentrations and the bacterial strains evaluated 2)

the efficiency of the disinfectant and growth inhibition bacterial, except in *Bacillus subtilis* strains (Table 1).

Table 1. In vitro inhibition of the aqueous extract of Neem leaves in the concentration 3, 4, and 5%

Treatment	<i>A. baumannii</i>	<i>B. subtilis</i>	<i>E. aerogenes</i>	<i>S. aureus</i>	<i>S. maltophilia</i>	<i>Micrococcus sp</i>	<i>S. epidermidis</i>
Neem 3%	99.84±0.04 b	0.00±0.00a	99.77±0.07 d	99.87±0.05 e	0.00±0.00 a	99.86±0.02 a	0.00±0.00a
Neem 4%	99.95±0.01 d	0.00±0.00a	99.75±0.02 d	99.85±0.02 d	0.00±0.00a	99.89±0.01 ab	0.00±0.00a
Neem 5%	100.00±0.0 0e	0.00±0.00a	99.85±0.02 e	99.96±0.01f	0.00±0.00a	99.97±0.01 d	0.00±0.00a
Enzymatic detergent 3%	99.81±0.01 a	0.00±0.00a	99.63±0.01 a	99.48±0.03 a	0.00±0.00a	99.88±0.00 a	99.69±0.01 b
Disinfectant 100%.	100.00±0.0 0e	0.00±0.00a	100.00±0.0 0f	100.00±0.0 0g	99.91±0.01 e	100.00±0.0 0d	100.00±0.0 0b

* According to Tukey 0.05, the values in a column followed by the same letter do not differ significantly.

Additionally, it should be noted that the Neem extract was effective in inhibiting the growth of five out of seven bacteria under study, at all concentrations used, being greater in 5 %, at 15 minutes of contact, and without significant differences with the other treatments (P=0.00). In the case of bacteria *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Staphylococcus aureus*, and *Micrococcus sp.*, inhibitions of 99.48% were observed, while in the case of *Bacillus subtilis*, *Stenotrophomonas maltophilia*, and *Staphylococcus epidermidis*, no bactericidal effect was observed by the Neem extract.

The *Bacillus subtilis* and *Stenotrophomonas maltophilia* strains were the most resistant; *Bacillus subtilis* was also not susceptible to the enzymatic detergent nor the disinfectant. *Stenotrophomonas maltophilia* also presented resistance to enzymatic detergent.

Discussion

This research indicated that using a single type of sanitizing product, applied for 15 minutes, is not efficient enough for reducing all bacteria genus that cause infections associated with health care, which were isolated from hospital's surfaces in the city of

Valledupar. This finding highlights the importance of trials that allow the proper selection and suitable disinfectants use in hospital areas that facilitate the construction of a clear policy to prevent HAIs (16).

Furthermore, regarding the *Bacillus subtilis* resistance to disinfectants, it is associated with its characteristics as a sporulated microorganism, which forms a barrier that prevents the antimicrobial agents' entrance since the complex membranes that surround the endospore act as an additional penetration factor (17). Besides, an interference with the culture medium could occur, and in the case of chemical products, a more prolonged action time is recommended.

The 99.96% effectiveness of the natural extract based on Neem leaves in concentrations of 3, 4, and 5% coincides with similar studies on *Staphylococcus aureus* isolated from cows milk with subclinical mastitis, where Giraldo (18) pointed out an 83% bacteriostatic activity of Neem extracts at a 5% concentration.

Valle (19) stated that the Neem leaf infusion is 100% effective against *Oxyuris equi*, at 15% concentration when used in oral administration of 60 ml. On the other hand, according to Dublin, Roque, and Estrada (20), several applications of Neem were required to achieve adequate effectiveness as an anthelmintic. Additionally, the treatments must be repeated to achieve cumulative effects, more significant bioactivity of the extract against bacteria *Staphylococcus epidermidis* and *Stenotrophomonas maltophilia*. In particular, those last emerging opportunistic pathogens with evidence of intrahospital transmission are resistant to multiple drugs and disinfectants, associated with their high mutagenic capacity (21,22). Likewise, in the study carried out by Vásquez (23), it was observed that the seed has a 40% bacterial inhibition and the leaves a 70%. Regarding *S. epidermidis*, it was 60%, and there was no inhibition of *S. aureus* and *E. coli*. Unlike the current study, Neem leaf's aqueous extract inhibited *S. aureus* (99.96%) and did not inhibit *S. epidermidis*. Variability in fact, according to the part of the plant used, indicates the need to standardize the extraction process to regulate the biocidal effect of the oil.

Similarly, López et al. (24) evaluated the microbicidal activity of acetonic, ethanolic and methanolic extracts of Neem seeds, at concentrations of 1, 10, 25 and 50%, against *Escherichia coli*, *Staphylococcus aureus*, and the Bacteriophage P22, using two contact times (2.5 and 5 min), reporting that the 10% ethanolic extract of Neem, inhibited the growth of *E. coli*. In comparison, the methanolic and acetonic extracts did so from the concentration of 25% and 50%, respectively, while the Neem extracts did not achieve a total reduction of *S. aureus*. This contrasting result may be due to the reduced contact time since the *S. aureus* inhibition was 15 minutes in the current study.

Conclusions

In this research, it was evidenced that the aqueous Neem extract has biocidal properties against bacteria associated with IAAS, which indicates its potential as an active ingredient of products for hospital use. To improve the Neem extract's effectiveness, a phytochemical study and

standardization of contact times for evaluation in a combined way on hospital surfaces are necessary.

Nevertheless, given the non-total effectiveness of any of the evaluated products, the combined use of them in a disinfection plan to control bacteria associated with IAAS is recommended.

Conflict of interests

The authors affirm that they have no conflict of interest with the publication of the results.

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Contribution of the authors

Hedilka Jiménez participated in the identification of bacteria associated with IAAS, Bertilda Pedraza prepared the neem extract, Jeraldin Castrillon and Aylin performed the sampling and biocidal test, Aslenis Melo performed the analysis of biocidal test data. All authors participated in the writing of the manuscript.